Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11349–11353, October 1996
Colloquium Paper

This paper was presented at a colloquium entitled "Genetic Engineering of Viruses and Virus Vectors," organized by Bernard Roizman and Peter Palese (Co-chairs), held June 9–11, 1996, at the National Academy of Sciences in Irvine, CA.

Applications of pox virus vectors to vaccination: An update

(vaccinia/fowlpox/canarypox/NYVAC vaccinia)

ENZO PAOLETTI

Delmar, NY 12054

ABSTRACT **Recombinant pox viruses have been generated for vaccination against heterologous pathogens. Amongst these, the following are notable examples. (*i*) The engineering of the Copenhagen strain of vaccinia virus to express the rabies virus glycoprotein. When applied in baits, this recombinant has been shown to vaccinate the red fox in Europe and raccoons in the United States, stemming the spread of rabies virus infection in the wild. (*ii*) A fowlpox-based recombinant expressing the Newcastle disease virus fusion and hemagglutinin glycoproteins has been shown to protect commercial broiler chickens for their lifetime when the vaccine was administered at 1 day of age, even in the presence of maternal immunity against either the Newcastle disease virus or the pox vector. (*iii*) Recombinants of canarypox virus, which is restricted for replication to avian species, have provided protection against rabies virus challenge in cats and dogs, against canine distemper virus, feline leukemia virus, and equine influenza virus disease. In humans, canarypox virus-based recombinants expressing antigens from rabies virus, Japanese encephalitis virus, and HIV have been shown to be safe and immunogenic. (*iv*) A highly attenuated vaccinia derivative, NYVAC, has been engineered to express antigens from both animal and human pathogens. Safety and immunogenicity of NYVAC-based recombinants expressing the rabies virus glycoprotein, a polyprotein from Japanese encephalitis virus, and seven antigens from *Plasmodium falciparum* have been demonstrated to be safe and immunogenic in early human vaccine studies.**

The notion that the work of Edward Jenner could be carried on after the successful global eradication of smallpox as a human infectious disease was provided by early descriptions of the engineering of vaccinia virus to express foreign genes (1, 2). Thus, by splicing genes from heterologous pathogens into the vaccinia virus vector one could immunize against that cognate pathogen. The 14 years since those publications were an exciting period where numerous strains of vaccinia were engineered to express a variety of antigens from a myriad of bacterial, viral, and parasitic pathogens with subsequent evaluation of the recombinants in both animal models as well as target species. Initial safety concerns of vaccinia virus vectors have been addressed by the use of highly attenuated replication-deficient strains of the virus as well as the engineering of host range-restricted pox viruses such as canarypox virus that, while restricted for productive replication to avian species, have been shown to effectively vaccinate nonavian targets. The initial studies on vaccinia virus were extended to other members of the pox virus family so as to provide species specific vectors. An example of this is the engineering of fowlpox-based vectors for use as recombinant vaccines in the poultry industry. Much information has been gained through this period and today some commercial success has been evidenced by the licensing of several products in the veterinary field. Today, in addition to continued work in this area for vaccines, pox virus-based vectors remain as eminent tools for studying the parameters of immune induction and new fields of endeavor are being investigated such as in cancer immunotherapy.

This paper will provide an update, albeit incomplete, of ongoing research with pox virus-based vectors.

Vaccinia-Rabies Glycoprotein G Recombinant

A vaccinia recombinant expressing the rabies virus glycoprotein was an early example of a successful pox virus vector useful in immunization (3). The vector was constructed by the insertion of the encoding cDNA for the rabies virus glycoprotein in the thymidine kinase locus of the Copenhagen strain of vaccinia virus. Disruption of the thymidine kinase locus allowed a biochemical selection of the recombinant as well as an attenuated phenotype to the vector. This recombinant has received a conditional commercial license in both Europe and in the United States.

The recombinant is administered as a live vaccine in baits for oral uptake by foxes in Europe and by raccoons in the United States. Extensive seeding of large geographic regions has provided field safety and efficacy. More recently, vaccine baits for controlling an epizootic of rabies in coyotes and grey fox in Texas has involved the seeding by air of more than 40,000 square miles with this recombinant vaccine.

Fowlpox Virus-Based Recombinants

The engineering of fowlpox virus-based vectors has direct application for recombinant vaccines in the poultry industry. Fowlpox virus is a pathogen in poultry. Attenuated fowlpox virus vaccines have been used for decades in the poultry industry to prevent wild-type virus infection. These attenuated fowlpox vaccine strains provide starting material for further construction of recombinant vaccines. The vector approach in poultry is confronted by issues similar to the general vaccine discipline and specifically to the vector approach. One such issue is how will preexisting maternal immunity influence the outcome of vaccination with a recombinant vector approach. In the poultry industry, this problem is generally twofold since the mother is immune to both the pathogen whose genes are to be expressed in the vector and to the fowlpox vector itself. The results of such a situation are detailed in ref. 5, where a fowlpox virus recombinant expressing the hemagglutinin neuraminidase and the fusion glycoproteins of Newcastle disease virus (NDV) are studied. A single inoculation in specific

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NDV, Newcastle disease virus; JEV, Japanese encephalitis virus; PRV, Pseudorabies virus; FeLV, feline leukemia virus; CTL, cytotoxic T lymphocyte.

Further, prior to the filing of applicants' application there was considerable uncertainty in the art regarding fowlpox vectors. For example, attached are copies of Paoletti, Proc. Natl. Acad. Sci. U.S.A, 93(21):11349-11353, 1996 and Taylor, Vaccine, 13(6):539-549, 1995. Both of these documents state that canarypox viruses have shown to be 100 times more efficient than a comparable fowlpox vector in inducing protective immunity (Paoletti, page 11350, second full paragraph and Taylor, page 539, passage bridging columns one and two). Accordingly, at the time of filing, one of ordinary skill in the art would not be motivated to select the claimed fowlpox virus vector from Paoletti.

In addition, Paoletti and Ramshaw do not provide any reasonable expectation that the claimed viral construct would be effective in inducing, enhancing or otherwise stimulating an immune response. The Examiner appears to consider that the prior art provides a general and broad expectation that co-expression of a cytokine with an antigen would enhance the immune response to an HIV antigen of interest (page 3, lines 9 et seq of the Office Action). The Examiner, however, has failed to identify why the combination of Paoletti and Ramshaw would have provided a reasonable expectation of success. Specifically, prior to the present invention, there had been no studies of co-expression (fowlpox-antigen-cytokine) in primates. As fowlpox poorly infects mammalian cells, there was a considerable risk that the vector would not work in primates. Further, at the time of filing, there was considerable uncertainty whether vectors which had shown to be immunogenic in mice would be immunogenic in primates. The technical data provided in the subject specification shows a vaccine construct according to the claims that is effective both in terms of its immunogenicity in primates and safety in primates. Without applicants' specification that provides proof of the effectiveness of the claimed viral construct, one of ordinary skill in the art would not be motivated to combine these specific references in the claimed manner to obtain a successful viral construct.

Accordingly, the Examiner has failed to provide the specific motivation required to combine the references. Further, the Examiner has failed to show that even if one of ordinary skill in the art were to have combined the disclosures of these references, they would have arrived at the claimed invention absent applicants' disclosure. Consequently, the rejections of claims 1, 17-19 and 38, should be withdrawn.

Claims 24, 25, 30 and 31 stand rejected under 35 USC 112, first paragraph, as not being enabled by the specification. In the interest of expediting prosecution, claims 24, 25, 30 and 31, have been cancelled without prejudice. Accordingly, this rejection is now moot.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **229752001400**.

Dated: September 13, 2006

Respectfully submitted,

By

Jonathan Bockman
Registration No.: 45,640
MORRISON & FOERSTER LLP
1650 Tysons Blvd, Suite 300
McLean, Virginia 22102
(703) 760-7769

pathogen-free birds at 1 day of age provided hemagglutinin-inhibiting antibodies that were maintained for the 8-week test period, which is the lifespan of a commercial broiler. Protective immunity was demonstrated against a combined intramuscular velogenic NDV challenge and a respiratory NDV challenge. Significantly, vaccination of commercial broiler chickens that retained a level of maternal immunity against both NDV and the vector resisted a subsequent challenge against both a lethal intramuscular NDV challenge, as well as a virulent fowlpox virus challenge. However, the NDV-specific immune response was at a reduced level. A fowlpox virus recombinant expressing NDV glycoproteins has received commercial licensure in the United States.

Avipox Virus Vectors in Nonavian Species

Members of the *Avipox* genus such as fowlpox and canarypox are distinguished by their host restriction for replication to avian species. It was discovered that inoculation of avipox-based recombinants into mammalian cells resulted in expression of the foreign gene and that inoculation into mammals resulted in the induction of protective immunity (6, 7). This surprising finding provided a significant safety profile to these vectors. Immunization could be affected in the absence of productive replication while eliminating the potential for dissemination of the vector within the vaccinate and, therefore, the spread of the vector to nonvaccinated contacts or to the general environment.

For reasons still not understood, it was demonstrated that a recombinant canarypox vector was a 100 times more efficient than a comparable fowlpox vector in inducing protective immunity and similar to a thymidine kinase-disrupted replication competent vaccinia virus vector (8).

Numerous examples have now been provided demonstrating the safety, immunogenicity, and protective efficacy of canarypox-based recombinants in both experimental animal models and target species. A prime example has used canarypox-based recombinants expressing the rabies virus glycoprotein G. Rabies virus infection and immunization are issues for both veterinary and human medicine. A great deal of information is available in rabies virus immunization, experimental animals and target species are readily available for study, and the parameters of successful immunization are understood. The safety and immunogenicity of a canarypox-based rabies glycoprotein recombinant was demonstrated in a number of nonavian species (9). Protection of vaccinated experimental animals or target species cats and dogs was demonstrated.

To appreciate the duration of immunity that could be engendered by vaccination with a canarypox-based recombinant, naive beagles were vaccinated by a single subcutaneous dose of the vaccine followed by rabies challenge with rabies virus. All vaccinated dogs seroconverted with maximal titers at 1 month. At various times after vaccination, a subset of dogs was challenged. At 6 and 12 months postvaccination, all dogs vaccinated with a single dose of the vaccine resisted challenge that was lethal to all the control animals. At 24 months after vaccination, 11 of 12 vaccinated dogs survived challenge with similar protection observed at 36 months postvaccination (10). These studies demonstrated that a single vaccination was immunogenic and that a protective immune response was primed such that recall as long as 3 years later was protective against a rabies virus challenge in the target species.

Successful vaccination in the presence of rabies-specific maternal antibodies was demonstrated in the following experiment using beagles. A worst scenario situation was established wherein pregnant bitches with immunity to rabies were revaccinated 2 weeks before whelping to maximize the antirabies antibody titers transferred from the bitch to the offspring. At 2 weeks after birth, the pups were vaccinated with a single dose of a canarypox-based rabies vaccine recombinant. Serological responses were followed to monitor either the decay of maternal antibodies in the nonvaccinated control pups or the effect on antibody titers on the pups vaccinated in the presence of maternal antibodies. At 3 months, immunity was challenged by inoculation of live rabies virus in the temporal muscle. The maternal antibody titer in the unvaccinated pups decayed with the expected kinetics. Pups vaccinated with the recombinant virus showed a slight increase in rabies virus neutralizing titer at 2 weeks postvaccination that fell to undetectable levels at the time of challenge. In a vaccine dose-dependent fashion, pups immunized in the presence of maternal immunity survived the rabies virus challenge that was lethal to all the nonvaccinated pups (10). This study demonstrated that young animals could be successfully vaccinated in the presence of maternal immunity.

The concept of using a nonreplicating avipox virus vector, a canarypox-based rabies recombinant, has been evaluated for safety and immunogenicity in human clinical studies (11, 12). Rabies naive healthy adult volunteers were inoculated with increasing doses of the recombinant in a schedule including a boost at 1 and 6 months. For comparison, the standard inactivated human diploid cell rabies vaccine was used. All inoculations with the recombinant canarypox vaccine were well-tolerated with only mild and short-lived reactions at the inoculation site reported. In these two clinical trials, induction of antirabies immune responses were demonstrated, and it was demonstrated that canarypox recombinants could be used either by themselves or in a protocol wherein the priming vaccination with the vector could be followed by a booster with the inactivated rabies vaccine.

Although the immune responses to the experimental canarypox recombinant were comparable but not demonstrated to be superior to those obtained with the standard inactivated rabies vaccine, it perhaps is not surprising given the relative low doses of the recombinant vaccine used in these studies and the comparison with an optimized and highly immunogenic licensed vaccine.

Other examples demonstrating the utility of canarypox virus-based vectors for veterinary species have been provided. Canarypox virus recombinants expressing the measles virus fusion and hemagglutinin glycoproteins have been used to vaccinate dogs. Comparison of these recombinants with vaccinia virus vectors expressing the same genes were shown to provide similar levels of immune response and protection against a challenge with the related Morbilli virus, canine distemper (13).

Construction of specific canine distemper virus recombinants expressing the fusion and hemagglutinin have been evaluated in the highly susceptible ferret model and dog host and were demonstrated to provide protection against challenge (unpublished data).

Canarypox-based recombinants expressing the hemagglutinin from equine influenza virus were shown to be immunogenic when inoculated in horses and provided protection against a naturally occurring equine influenza virus infection (14).

Two canarypox virus-based recombinants were constructed, each expressing the entire *gag* gene and either the intact subgroup A *envelope* of feline leukemia virus (FeLV) or a modified version of the *envelope* from which the putative immunosuppressive region was deleted (15). These recombinants were evaluated for protective efficacy in kittens of 8–9 weeks of age. Two inoculations of the recombinants at 5 and 2 weeks before challenge failed to induce measurable FeLV neutralizing antibodies. Nevertheless, 50% of the cats receiving the mutated *envelope* recombinant and 100% of the cats receiving the intact *envelope* recombinant were protected against an oronasal challenge with the FeLV-A/Glasgow-1 isolate. Protection was assessed by evaluating p27 antigenimea, detecting FeLV antigen in blood smears, and the attempted recovery of infectious FeLV. This was the first description of

a successful immunization against a retrovirus provided by pox virus-based recombinants.

The above observations provided an impetus to further investigate the potential of canarypox-based vectors for immunization against other retrovirus with particular attention on the lentiviruses with focus on HIV, the causative infectious agent of AIDS. The entire *envelope* protein of the human T-cell leukemia/lymphoma virus type I was expressed in a canarypox virus vector. Two inoculations of the recombinant vaccine candidate were administered to rabbits. Five months after the last inoculation, the animals were exposed to a human T-cell leukemia type-I cell associated challenge from a primary culture of the *bou* isolate. The animals were protected. The protected animals were again challenged 5 months after the initial challenge exposure with 5 ml of blood from an infected rabbit. Immunity failed this relatively large challenge exposure. Of interest in these studies (16) was the observation that if a subunit *envelope* booster was administered in alum after the priming vaccination with the canarypox recombinant protection was not obtained. Interpretation of this observation can lead to interesting speculation.

Other interesting observations using canarypox-based recombinants expressing antigens from either HIV-I or II, as well as simian immunodeficiency virus, have been reported. In laboratory rodents, induction of both humoral immunity as well as cytotoxic T lymphocyte (CTL) can readily be demonstrated (17).

Recombinants expressing HIV-II *gag*, *pol*, or *envelope* genes have been evaluated in macaques in several studies with some level of protection described (18, 19). Significant and raising concerns for those involved in vaccine development correlates of protective immunity are not revealed in these studies. Multiple immunization allowing for the maturation of the immune response is suggested by some studies (20). An intriguing observation was the cross protection against HIV-II challenge in monkeys vaccinated with HIV-1 recombinant pox viruses (21). A likely interpretation of this data is the induction of and protection by cross-reactive CTL. However, the basis of this cross protection is currently unknown.

A series of recombinant canarypox virus-based recombinants expressing an increasing complexity of HIV-I strain MN antigens have been constructed and evaluated in human clinical trials for both safety and immunogenicity. The earliest of these studies in HIV seronegative healthy adult volunteers have been reported (22). A vaccine regimen providing the best results to date involve one or two doses of the recombinant canarypox virus vector followed by one or two doses of an adjuvanted recombinant *envelope* subunit. The induction of binding, HIV neutralizing, and both CD4 and CD8 CTL have been reported (22–24).

More recent data using the more complex recombinants and higher doses of vaccine in a vector prime/subunit antigen boost protocol have demonstrated better levels of neutralizing antibody induction and a more complex reactivity of CTL to multiple HIV antigens. Further comparison of separate phase I trial data a prime/boost protocol using the canarypox vector fares favorably when compared with a prime boost protocol using a replication competent vaccine vector as a primer (unpublished data). In this light, the failure of the canarypox vector to replicate in the mammalian host provides advantage over the replication competent vaccinia virus vector. The general safety profile of the HIV-I canarypox recombinants in human volunteers is similar to that observed with the canarypox recombinants expressing the rabies virus glycoprotein discussed above.

Attenuated Vaccinia-Based Vector: NYVAC

The global smallpox eradication program was made possible by several biological features of the pathogen and the vaccine. The pathogen had only a single host for infection and propagation—man. There were no animal reservoirs from which the pathogen could recrudesce. Defined outbreaks of the infection could be circumscribed and contained by vaccination. Vaccinia, the vaccine, could be produced efficiently and at low cost in regional centers. The ability to retain potency of the vaccine as a freeze-dried preparation allowed storage and transport to remote regions of the globe. The successful smallpox eradication program, however, was not without vaccine-associated risk. Vaccine reactogenicity with some severe or lethal outcomes was associated with the vaccine in general and specifically higher rates of adverse events were evidenced in certain populations or with certain vaccine strains or preparations. Early attempts to manufacture the vaccine under more defined and regulated laboratory conditions were abandoned with the success of the eradication effort. The known reactogenicity of the vaccinia vaccine was therefore a concern to be addressed when the virus was proposed as a vector for new engineered vaccines. This concern has been addressed in several ways such as the provision of naturally host-restricted vectors described above or by the targeted attenuation of existing vaccine strains. This approach is demonstrated by the engineering of the NYVAC strain of vaccinia virus. The Copenhagen strain of vaccinia was chosen as a vaccine substrate. The entire DNA sequence of the genome was established (25). With this information and the extant knowledge of virulence-related and other genetic functions related to host range replication competency unwanted genetic information was precisely deleted from the vaccinia virus genome. The resultant vector, NYVAC, was highly attenuated as demonstrated in a series of studies in animal surrogates (26). Intracranial inoculation of newborn or young adult mice demonstrated a very favorable dose range compared with either the parent or other vaccine strains, and significantly no disseminated viral infection was observed in immunocompromised hosts. In numerous tissue culture cells of human origin, the vector was shown to be highly debilitated for replication consistent with the deletion of host range genes. The modified NYVAC vector, while highly attenuated, retained the ability to induce protective immune responses to foreign antigens in a fashion similar to the thymidine kinase mutant of the parent strain.

A number of examples using the NYVAC vector as a recombinant vaccine delivery system have been provided in animal model systems and in target species including humans. A series of NYVAC recombinants were generated to express glycoproteins from Pseudorabies virus (PRV) and the immunity afforded by these recombinants was evaluated in the target species of PRV infection, the pig. PRV neutralizing antibodies were induced following two intramuscular inoculations 28 days apart. The NYVAC recombinant expressing the PRV glycoprotein gp50 induced levels of PRV neutralizing antibodies and afforded protection against a virulent oronasal PRV challenge that was comparable to vaccination with inactivated PRV vaccine (27). The advantage of a recombinant vaccine is that one is allowed to discriminate between a naturally infected versus vaccinated animal since the recombinant vaccine displays a defined subset of the antigens of the pathogens. This discrimination allows the agricultural industry to properly track infections and cull infected herds.

A NYVAC-based recombinant expressing two hemagglutinin glycoproteins of the A1 and A2 equine influenza serotypes induced hemagglutinin inhibiting antibodies when inoculated into horses and afforded significant protection when the vaccinated horses became exposed to a natural equine influenza virus infection (14).

The polyprotein of Japanese encephalitis virus (JEV) encoding prM/M, E, and NS1 was expressed in NYVAC recombinants and the vector used to vaccinate swine, a major natural host of JEV infection and a reservoir for mosquito transmis-

sion of the virus to man. Hemagglutinin-inhibiting and JEV-neutralizing antibodies were induced on vaccination. The nonvaccinated challenged animals succumbed to JEV infection, whereas the vaccinated group had levels of JEV challenge viremia insufficient to be transmitted by mosquitoes (28) Both a NYVAC- and a canarypox-based Japanese encephalitis recombinant are currently being evaluated in human clinical trials.

A NYVAC vector has been engineered to express the rabies glycoprotein gene. In mice, cats, and dogs, the recombinant was shown to be safe and to provide protection against a lethal rabies virus challenge. The recombinant is now being evaluated in phase I human clinical trials for safety and immunogenicity.

Pox virus vectors have been used to determine the immunogenic potential of antigens from *Plasmodium* spp. in an effort to understand the design of an effective vaccine against malarial infections. In this regard a NYVAC vector reconstituted with the K1L host range gene was constructed to express intact or mutated forms of the circumsporozoite protein of *Plasmodium berghei*. Vaccination of the target host, the mouse, induced both binding antibody and CTL. Vaccinated and control mice were challenged either by the intravenous injection of sporozoites or by allowing infected mosquitoes to feed on the subjects. Protection was scored as the absence of blood stage parasetemia as determined by microscopic analysis of blood films from individual mice from 5–15 days after challenge. In a number of challenge experiments, ~80% protection was obtained. This is to be compared with the consistent 100% level of protection obtained by vaccination with irradiated sporozoites. Protection in the recombinant virus-immunized mice apparently did not correlate with antibodies but a good correlation was established between CTL and protection. *In vivo* antibody depletion of $CD8^+$ T cells before challenge abrogated protection (29).

With this data as an inducement, a complex NYVAC-based recombinant was constructed to express multiple antigens from *P. falciparum*. To address the multiple stages of the parasite life cycle, multiple antigens from the various stages were used. Thus, a recombinant expressing seven parasite antigens was provided. This recombinant was evaluated in rodents and in monkeys where safety and immunogenicity were established (30). This recombinant is now being evaluated in clinical trials where the vaccinated subjects are exposed to the bites of infected mosquitoes. Appearance of parasites in the blood of the infected volunteers will terminate the challenge followed by administration of antimalarial drugs to thwart further replication of the parasite. Since ethical and medical considerations require treatment on appearance of blood-stage parasites, only the antisporozoite and liver-stage immunity engendered by the vaccine can be evaluated. Full evaluation of blood-stage and transmission-blocking immunity cannot be evaluated in this limited clinical setting.

To date, all the above-mentioned abstracted data provided from human clinical trials using NYVAC-based vectors have described a good safety profile and the induction of some level of immunity to the expressed heterologous antigens.

Other Applications of Pox Virus-Based Vectors

The use of pox virus-based vectors as recombinant vaccines for heterologous bacterial, viral, or parasitic pathogens was the first practical application of this technology deriving from the fact that vaccinia virus was an established vaccine. However, the pox virus vectors can be looked at as general delivery systems for genes for other applications. For example, these vectors can be used *in vitro* to stimulate and expand CTL reactivities from the peripheral blood of chronically infected or tumor-bearing individuals (31). The antigen-specific stimulation and expansion of such cultures might provide some therapeutic benefit when reintroduced to the donor patient.

For cancer immunotherapy, numerous pox virus-based recombinants expressing tumor-associated antigens or biological response modifiers have been described (32). Of particular note, recombinants expressing the carcinoembryonic antigen were shown to elicit both antibody and cellular immune responses in mice and monkeys and to protect mice from tumor cell challenge (33, 34). Whether vaccinia or canarypox-based recombinants expressing the carcinoembryonic antigen will have any therapeutic benefit is currently being investigated in the clinic in patients with colorectal carcinomas.

A recent publication (4) reported the protection of mice vaccinated with a p53 expressing recombinant against challenge with an isogenic and highly tumorigenic mouse fibroblast tumor cell line expressing high levels of a mutant human p53 but lacking endogenous murine p53. Expression of the mutant form of p53 in the recombinant virus was not essential since the wild-type p53 afforded similar efficacy. This may be an important observation since p53 is an attractive target for cancer immunotherapy. Mutations of p53 represent the most common genetic changes demonstrated in human tumors.

Discussion

The excitement of the 1982 proposal to use pox virus-based vectors as heterologous vaccines and the ensuing years of extensive pursuit of this idea have provided numerous working examples in laboratory animal model systems as well as in target species. In the veterinary field, products have now been licensed for commercialization and a significant number of clinical studies have been and continue to be pursued for both infectious diseases, *ex vivo* therapies, and cancer immunotherapy. The immediate future looks to be as exciting as the recent past.

1. Panicali, D. & Paoletti, E. (1982) *Proc. Natl. Acad. Sci. USA* 79, 4927–4931.
2. Mackett, M., Smith, G. L. & Moss, B. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7415–7419.
3. Kieny, M. P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H. & Lecocq, J. P. (1984) *Nature (London)* 312, 163–166.
4. Roth, J., Dittmer, D., Rea, D., Tartaglia, J., Paoletti, E. & Levine, A. J. (1996) *Proc. Natl. Acad. Sci. USA* 93, 4781–4786.
5. Taylor, J., Christensen, L., Gettig, R., Goebel, J., Bouquet, J.-F., Mickle, T. R. & Paoletti, E. (1996) *Avian Dis.* 40, 173–180.
6. Taylor, J. & Paoletti, E. (1988) *Vaccine* 6, 466–468.
7. Taylor, J., Weinberg, R., Languet, B., Desmettre, P. & Paoletti, E. (1988) *Vaccine* 6, 497–503.
8. Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, P. & Paoletti, E. (1991) *Vaccine* 9, 190–193.
9. Taylor, J., Meignier, B., Tartaglia, J., Languet, B., VanderHoeven, J., Franchini, G., Trimarchi, C. & Paoletti, E. (1995) *Vaccine* 13, 539–549.
10. Taylor, J., Tartaglia, J., Rivière, M. & Paoletti, E. (1994) *Dev. Biol. Stand.* 82, 131–135.
11. Cadoz, M., Strady, A., Meignier, B., Taylor, J., Tartaglia, J., Paoletti, E. & Plotkin, S. (1992) *Lancet* 339, 1429–1432.
12. Fries, L. F., Tartaglia, J., Taylor, J., Kauffman, E. K., Meignier, B., Paoletti, E. & Plotkin, S. (1996) *Vaccine* 14, 428–434.
13. Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E. & Paoletti, E. (1992) *Virology* 187, 321–328.
14. Taylor, J., Tartaglia, J., Moran, T., Webster, R. G., Bouquet, J.-F., Quimby, F. W., Holmes, D., Laplace, E., Mickle, T. & Paoletti, E. (1992) *Proceedings of the Third International Symposium on Avian Influenza* (Univ. of Wisconsin Extension Duplicating Serv., Madison), pp. 311–335.
15. Tartaglia, J., Jarrett, O., Neil, J. C., Desmettre, P. & Paoletti, E. (1993) *J. Virol.* 67, 2370–2375.
16. Franchini, G., Tartaglia, J., Markham, P., Benson, J., Fullen, J., Wills, M., Arp, J., Dekaban, G., Paoletti, E. & Gallo, R. C. (1995) *AIDS Res. Hum. Retroviruses* 11, 307–313.
17. Cox, W. I., Tartaglia, J. & Paoletti, E. (1993) *Virology* 195, 845–850.

18. Franchini, G., Robert-Guroff, M., Tartaglia, J., Aggarwal, A., Abimiku, A., Benson, J., Markham, P., Limbach, K., Hurteau, G., Fullen, J., Aldrich, K., Miller, N., Sadoff, J., Paoletti, E. & Gallo, R. C. *AIDS Res. Hum. Retroviruses* **11**, 909–920.
19. Andersson, S., Makitalo, B., Thorstensson, R., Franchini, G., Tartaglia, J., Paoletti, E., Putkonen, P. & Biberfeld, G. I (1996) *J. Infect. Dis.*, in press.
20. Myagkikh, M., Alipanah, S., Markham, P. D., Tartaglia, J., Paoletti, E., Gallo, R. C., Franchini, G. & Robert-Guroff, M. (1996) *AIDS Res. Hum. Retroviruses* **12**, 985–992.
21. Abimiku, A. G., Franchini, G., Tartaglia, J., Aldrich, K., Myagkikh, M., Markham, P. D., Chong, P., Klein, M., Kieny, M.-P., Paoletti, E., Gallo, R. C. & Robert-Guroff, M. (1995) *Nat. Med.* **1**, 321–329.
22. Pialoux, G., Excler, J.-L., Rivière, Y., Gonzalez-Canali, G., Feuillie, V., Coulaud, P., Gluckman, J.-C., Matthews, T. J., Meignier, B., Kieny, M.-P., Gonnet, P., Diaz, I., Méric, C., Paoletti, E., Tartaglia, J., Salomon, H., Plotkin, S., and The AGIS Group and L'Agence Nationale de Recherche sur le SIDA (1995) *AIDS Res. Hum. Retroviruses* **11**, 373–381.
23. Egan, M. A., Pavlat, W. A., Tartaglia, J., Paoletti, E., Weinhold, K. J., Clements, M.-L. & Siliciano, R. F. (1995) *J. Infect. Dis.* **171**, 1623–1627.
24. Fleury, B., Janvier, G., Pialoux, G., Buseyne, F., Robertson, M. N., Tartaglia, J., Paoletti, E., Kieny, M. P., Excler, J. L. & Rivière, Y. (1996) *J. Infect. Dis.*, in press.
25. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P. & Paoletti, E. (1990) *Virology* **179**, 247–266.
26. Tartaglia, J., Perkus, M. E., Taylor, J., Norton, E. K., Audonnet, J.-C., Cox, W. I., Davis, S. W., VanderHoeven, J., Meignier, B., Rivière, M., Languet, B. & Paoletti, E. (1992) *Virology* **188**, 217–232.
27. Brockmeier, S. L., Lager, K. M., Tartaglia, J., Rivière, M., Paoletti, E. & Mengeling, W. L. (1993) *Vet. Microbiol.* **38**, 41–58.
28. Konishi, E., Pincus, S., Paoletti, E., Laegreid, W. W., Shope, R. E. & Mason, P. W. (1992) *Virology* **190**, 454–458.
29. Lanar, D. E., Tine, J. A., de Taisne, C., Seguin, M. C., Cox, W. I., Winslow, J. P., Ware, L. A., Kauffman, E., Gordon, D., Ballou, W. R., Paoletti, E. & Sadoff, J. C. (1996) *Infect. Immun.* **64**, 1666–1671.
30. Tine, J. A., Lanar, D. E., Smith, D., Wellde, B. T., Schultheiss, P., Ware, L. A., Kauffman, E., Wirtz, R. A., de Taisne, C., Hui, G. S. N., Chang, S. P., Church, P., Kaslow, D. C., Hoffman, S., Guito, K. P., Ballou, W. R., Sadoff, J. C. & Paoletti, E. (1996) *Infect. Immun.*, in press.
31. Tartaglia, J., Taylor, J., Cox, W. I., Audonnet, J.-C., Perkus, M. E., Radaelli, A., de Giuli Morghen, C., Meignier, B., Rivière, M., Weinhold, K. J. & Paoletti, E. (1993) *AIDS Res. Rev.* **3**, 361–378.
32. Perkus, M. E., Tartaglia, J. & Paoletti, E. (1995) *J. Leukocyte Biol.* **58**, 1–13.
33. Kantor, J., Irvine, K., Abrams, S., Kaufman, H., DiPietro, J. & Schlom, J. (1992) *J. Natl. Cancer Inst.* **84**, 1084–1091.
34. Kantor, J., Irvine, K., Abrams, S., Snoy, P., Olsen, R., Greiner, J., Kaufman, H., Eggensperger, D. & Schlom, J. (1992) *Cancer Res.* **52**, 6917–6925.

HEINEMANN

0264-410X(94)00028-X

Vaccine, Vol. 13, No. 6, pp. 539-549, 1995

0264-410X/95 $10.00+0.00

Biological and immunogenic properties of a canarypox–rabies recombinant, ALVAC-RG (vCP65) in non-avian species

J. Taylor*, B. Meignier†, J. Tartaglia*, B. Languet‡, J. VanderHoeven*, G. Franchini§, C. Trimarchi‖ and E. Paoletti*

A canarypox-based (ALVAC) recombinant expressing the rabies G glycoprotein has been utilized to assess in vitro *and* in vivo *biological properties of the canarypox virus vector system.* In vitro *studies have shown that no replication of the virus can be detected on six human-derived cell lines, nor can the virus be readily adapted to replicate on non-avian cells. Expression of the rabies G can be detected on all cell lines analyzed in the absence of productive viral replication. Analysis of viral-specific DNA accumulation indicated that the block in the replication cycle in the human cell lines analyzed occurred prior to DNA replication. The exact nature of the block, however, remains unknown. The concept of using a non-replicating immunization vehicle has been demonstrated through extensive* in vivo *studies in a range of species including non-human primates and humans. The results of such* in vivo *studies have exemplified the safety and immunogenicity of the ALVAC vaccine vector.*

Keywords: Poxvirus-based vaccines; canarypox virus (ALVAC); ALVAC-RG(vCP65); safety; immunogenicity

The development of naturally host-restricted avipox virus vectors capable of expressing extrinsic immunogens and inducing a protective immune response against lethal viral challenge in mammalian species has been described[1-5]. Fowlpox virus (FPV) and canarypox virus (CPV) are members of the avipox virus genus of the Orthopoxvirus family. Productive replication of avipox viruses is restricted to avian species[6]. Both FPV and CPV-rabies recombinants[1,2] express the rabies glycoprotein in tissue culture cells of non-avian origin without apparent replication of the vector virus. Inoculation of these recombinants into a range of non-avian species including mice, cats, and dogs demonstrated that the level of expression of the foreign gene product was sufficient to induce rabies-specific serum neutralizing antibodies and to protect against a lethal rabies virus challenge.

Potency tests in mice indicated that a CPV vector expressing the rabies glycoprotein was 100-fold more efficacious than an FPV-based vector and that the protective efficacy of a host-restricted CPV-rabies vector was similar to that of a replication-competent vaccinia virus vector containing the rabies G gene in the thymidine kinase locus[2]. Further, both replication competent VV-measles recombinants and a host-restricted CPV-measles recombinant induced similar levels of measles virus neutralizing antibody and protection against experimental canine distemper virus challenge in dogs[3].

Additional studies have shown that the utility of avipox vectors as immunizing agents in non-avian species is not limited to the rabies glycoprotein or measles virus immunogens. Vaccination of cats with an ALVAC-based recombinant expressing the feline leukemia virus (FeLV) A subtype Env and Gag proteins protects against the development of persistent viremia following FeLV challenge exposure[7]. ALVAC recombinants expressing immunogens from Japanese encephalitis virus (JEV) have also been shown to protect mice against a lethal JEV challenge[8]. Safety and immunogenicity studies in horses utilizing an ALVAC recombinant expressing the hemagglutinin glycoproteins from the A1 and A2 serotypes of equine influenza virus demonstrated the induction of type specific hemagglutination-inhibiting antibodies and protection against an A2 epizootic[4]. An ALVAC-based recombinant expressing the HIV-1 envelope glycoprotein has recently been shown to induce HIV-specific antibody and cytotoxic T-lymphocyte responses in mice[9]. These examples of ALVAC recombinants expressing immunogens from a variety of viral pathogens indicate the general utility of ALVAC-based

*Virogenetics Corporation, Rensselaer Technology Park, 465 Jordan Road, Troy, NY 12180, USA. †Pasteur Merieux Serums et Vaccins, 1541, Avenue Marcel Merieux, 69280 Marcy L'Etoile, France. ‡Rhone Merieux, 254 rue Marcel Merieux, Lyon, Cedex 07, France. §Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. ‖Griffin Laboratories, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201, USA.
(Received 2 May 1994; revised 17 October 1994; accepted 17 October 1994)

recombinant viruses as immunization vehicles in a variety of mammalian species.

Avipox virus-based vectors theoretically provide significant safety advantages in light of their inability to productively replicate in non-avian species. Such a vaccine vector should not allow dissemination within the vaccinated individual, contact transmission to non-vaccinated individuals, or general contamination of the environment. The concept of utilizing a replication-restricted vaccine vector, ALVAC-RG, in humans has recently been assessed with promising results. The experimental vaccine was well tolerated and induced protective levels of rabies-neutralizing antibodies[10, 11].

These studies in target species have provided a database which has indicated that ALVAC-based recombinants may have significant advantages as vector-based vaccines. It therefore was critical to rigorously evaluate the safety characteristics of ALVAC-based recombinants in laboratory animals and to establish the innocuity and immunogenicity of the vector in both non-human primates and target species. This report describes *in vitro* and *in vivo* studies designed to explore the basis of host restriction, safety, and immunizing potential of the ALVAC-based vector.

MATERIALS AND METHODS

Cells and viruses

Viral amplifications and plaque titrations were performed on primary chicken embryo fibroblast (CEF) cells from 10 to 11 day embryos of SPF origin. Titrations were also performed as a microtiter assay on the quail QT35 cell line[12] and titers determined by the method of Karber[13].

The origin of other cells used in this study is as follows: (1) VERO cells (ATCC No. CCL81) are a line derived from African Green Monkey kidney; (2) MRC-5 (ATCC No. CCL171) are of human embryonic lung origin; (3) HNK are human neonatal kidney cells subcultured for less than five passages (Whittaker BioProducts, Inc., Walkersville, MD (Cat. No. 70-151)); (4) HEL 299 are human embryonic lung cells (ATCC No. CCL137); (5) WISH are of human amnion origin (ATCC No. CCL25); (6) DETROIT 532 are of human foreskin (Down's Syndrome) origin (ATCC No. CCL54); (7) JT-1 is a human lymphoblastoid cell line transformed with Epstein-Barr virus as described in Rickinson *et al.*[14]

The canarypox virus strain from which ALVAC was derived was isolated from a pox lesion on an infected canary. The virus was first isolated at the Rentschler Bakteriologishes Institut, Lauphein, Wurtemberg, Germany, where it was attenuated by 200 serial passages in CEFs. This attenuated strain (Kanapox) obtained from Rhone Merieux is licensed as a vaccine for canaries in France. At Virogenetics, the virus was subjected to four successive rounds of plaque purification under agarose. One plaque isolate, designated ALVAC, was selected for amplification and used in these studies.

Development of recombinant ALVAC-RG (vCP65)

The canarypox rabies recombinant was derived by methods previously described[1, 15, 16]. A unique insertion locus was defined at a *Bgl*II site within an 880 bp *Pvu*II fragment of CPV genomic DNA. The DNA sequence of this fragment was determined and the open reading frame (ORF) designated as C5 defined. Deletion of the entire C5 ORF was made by standard molecular biological procedures[17, 18] without interruption of neighboring ORFs. The C5 ORF was replaced by *Hind*III, *Sma*I and *Eco*R1 insertion sites followed by translation termination codons and early vaccinia virus transcription termination signals[19].

The ERA strain rabies glycoprotein cDNA[20, 21] linked to the early/late vaccinia virus H6 promoter[1, 16, 22] was inserted at the *Sma*I site. The resulting plasmid, pRW838, was transfected into ALVAC-infected primary CEF cells using the calcium phosphate precipitation method[15]. Plaques were selected on the basis of DNA hybridization to a rabies G-specific radiolabelled probe and subjected to sequential rounds of plaque purification. A representative plaque was then amplified and designated ALVAC-RG with the laboratory designation of vCP65.

Inoculation of non-avian cells with ALVAC-RG

A variety of human cell substrates, MRC-5, HNK, HEL, DETROIT-532, WISH and JT-1, were inoculated with ALVAC-RG and analyzed for expression of the rabies G gene, and accumulation of viral-specific DNA. Primary CEF cells were included as a permissive substrate.

Viral DNA accumulation

Sixty millimetre dishes containing two million cells of each cell type under test were inoculated with ALVAC at a multiplicity of infection (MOI) of 5 p.f.u. per cell. After an adsorption period of 1 h at 37°C, the inoculum was removed, the monolayer washed twice to remove unadsorbed virus and the infected monolayer refed with 5 ml of Eagle's Minimal Essential Medium (EMEM) + 2% Newborn Calf Serum (NCS). Cells from one dish were harvested at t_0 and the remaining dishes were incubated, in the presence or absence of 40 μg ml^{-1} of cytosine arabinoside (AraC; Sigma No. C6654), at 37°C for 72 h. Cells were collected and resuspended in 0.5 ml phosphate buffered saline (PBS) containing 40 mM EDTA and incubated for 5 min at 37°C. An equal volume of 1.5% agarose containing 120 mM EDTA, prewarmed to 42°C, was gently mixed with the cell suspension and transferred to an agarose plug mold. After solidification, the agarose plugs were removed and incubated for 12–16 h at 50°C in a volume of lysis buffer (1% sarkosyl, 100 μg ml^{-1} proteinase K, 10 mM Tris HCl pH 7.5, 200 mM EDTA) sufficient to cover the plug. The lysis buffer was then replaced with 5 ml 0.5×TBE (44.5 mM Tris borate, 44.5 mM boric acid, 0.5 mM EDTA) and equilibrated at 4°C for 6 h with 3 changes of 0.5×TBE buffer. The viral DNA within the plug was fractionated from cellular nucleic acid using a BIO-RAD CHEF-DR II pulse field electrophoresis system (180 V/20 h/15°C) in 0.5×TBE with a ramp time of 50–90 s, using lambda DNA as molecular weight standards. The viral DNA band was first visualized by staining with EtBr, then transferred to a nitrocellulose membrane and probed with a radiolabelled probe prepared from purified canarypox genomic DNA.

Analysis of expression of rabies G gene

Immunoprecipitation analysis was performed from a radiolabelled lysate of each cell line infected with ALVAC or ALVAC-RG (vCP65) as described in Tartaglia *et al.*[18] using a rabies glycoprotein-specific monoclonal antibody designated 24-3F10.

Time course study

MRC-5 and CEF monolayers were inoculated with 10 pfu/cell of ALVAC or ALVAC-RG (vCP65) at 37°C for 60 min. The inoculum was removed, the monolayer washed twice, and the medium replaced. At 1, 3, 5, 9, 13, 17, and 25 h post infection, the culture was labelled for 1 h by the addition of methionine-free medium containing 25 μCi ml^{-1} of ^{35}S-methionine (DuPont NEN; 1140 Ci mmol^{-1}). Infected cells were scraped from the culture dishes, collected by centrifugation, washed twice with PBS and lysed by the addition of 2 ml of Buffer A (18). Infected cell lysates were analyzed for expression of the rabies G gene by immunoprecipitation as described in Tartaglia *et al.*[18]

Safety studies in laboratory animals

Groups of rabbits (New Zealand white ESD), guinea pigs (Dunkin-Hartley, Libeau) and mice (IFFA Credo, Les Oncins, France) were inoculated with ALVAC-RG by a variety of routes as shown in *Table 1*. Animals were inspected daily for signs of reactogenicity and at the termination of the test at 14–21 days, animals were euthanized and tissue at the site of inoculation examined. To monitor neurovirulence, nine male OF$_1$ mice were anesthetized and injected by the i.c. route with ALVAC-RG (vCP65). Three mice were inoculated with an uninfected cell extract. Three inoculated and one control mice were sacrificed on days 1, 3 and 6 post-inoculation. Brains were fixed *in situ* by immersing the opened skull in a solution of buffered formalin. After processing, 5 sections were made and stained with gallocyanin/phloxine. The sections involved the following levels: A: corpus striatum, B: infundibulum, C: pedunculi cerebri, D: pons and E: cerebellum.

Comparison of virulence of Kanapox virus (Rentschler strain of CPV) and ALVAC-RG in canary birds

Canary birds certified to have not been immunized with canarypox virus were obtained from PIC Grains (Vignouse sur Barangeon, France). Birds were inoculated with 5.0 or 7.0 log$_{10}$ p.f.u. of either Kanapox (Rentschler strain of CPV) or ALVAC-RG (vCP65) by

Table 1 Safety studies in laboratory animals: schedule of inoculation by different routes

Species	Virus	Dose	Route	Volume	Sites
Rabbit	ALVAC	5.7[a]	i.c.	0.1 ml	1
	ALVAC-RG	5.7	i.c.	0.1 ml	1
	ALVAC-RG	6.3	i.d.	0.2 ml	5
	ALVAC-RG	8.0	s.c.	9.0 ml	1
Guinea pig	ALVAC-RG	6.0	i.d.	0.1 ml	5
	ALVAC-RG	7.3	s.c.	2.0 ml	1
Mice	ALVAC-RG	5.7	i.d.	0.05 ml	5
	ALVAC-RG	6.7	s.c.	0.5 ml	1
	ALVAC-RG	6.0	i.c.	0.05 ml	1

[a]Inoculum dose expressed as log$_{10}$TCID$_{50}$

smearing 50 μl of a 1:1 mixture of virus suspension and glycerin on a 0.5 cm^2 area from which the feathers had been removed on the back of each bird. Birds were monitored on a daily basis for one month postinoculation with weighing at 2–3 day intervals.

Inoculation of ALVAC-RG into the skin of canary birds and mice

Female OF$_1$ mice were injected by the i.d. route in each ear pinna with 5.0 log$_{10}$ TCID$_{50}$ of ALVAC-RG (vCP65) in 20 μl. Canary birds received an equivalent dose mixed with glycerin and smeared on a 1.0 cm^2 area of skin on the back from which feathers had been removed. At time intervals, animals were sacrificed and the skin surrounding the site of inoculation was dissected, homogenized in medium 199/Ham F10 plus 2% FCS, and stored at −70°C. Mouse specimens consisted of the entire skin covering the dorsal face of the ear pinna. Homogenates were thawed, sonicated, centrifuged, diluted 1:100 to avoid toxicity, and titrated in serial dilutions in QT35 cells.

Immunogenicity and safety studies in primate species

Three species of non-human primate, rhesus macaques, chimpanzees and squirrel monkeys (*Saimiri sciureus*) were inoculated with ALVAC-RG as shown in *Table 2*. The study in squirrel monkeys also addressed the questions of the ability to re-isolate virus after inoculation by a variety of routes and the immune response to ALVAC-RG in the face of pre-existing ALVAC immunity. In this study, three groups of four squirrel monkeys were inoculated with one of three viruses: (a) ALVAC, the parental canarypox virus; (b) ALVAC-RG (vCP65); or (c) ALVAC-FL (vCP37), a canarypox recombinant expressing the envelope glycoprotein of FeLV (Tartaglia *et al.*, unpublished data). Inoculations were performed under ketamine anesthesia. Each animal received at the same time: (1) 20 μl instilled on the surface of the right eye without scarification; (2) 100 μl as several droplets in the mouth; (3) 100 μl in each of two i.d. injection sites in the shaven skin of the external face of the right arm; and (4) 100 μl in the anterior muscle of the right thigh. In each group, two animals received 5.0 log$_{10}$ p.f.u. and two animals received 7.0 log$_{10}$ p.f.u. of the appropriate virus. Virus isolation was attempted from the site of inoculation for 11 days post-inoculation and all monkeys were monitored for adverse reactions. Six months after the initial inoculation, selected animals from each group plus one canarypox naive animal were inoculated with ALVAC-RG (vCP65) as described in *Table 2*. All animals were monitored for adverse reactions to vaccination and sera analyzed for the presence of anti-rabies antibodies[24].

RESULTS

Derivation of ALVAC-RG (vCP65)

The strategy used to develop FPV[1,16,23] and CPV[2,3] recombinants involved insertion of the foreign gene at a unique restriction site within an ORF previously defined as nonessential. No attempt was made to precisely delete the interrupted ORF. In the generation of ALVAC-RG (vCP65), an insertion plasmid containing the H6/rabies G expression cassette was constructed

Table 2 Schedule of inoculation of primate species with ALVAC-RG (vCP65)

Species	Designation	Dose[a]	Route	Previous inoculations	Booster dose, interval
Rhesus macaque	177 and 186	7.7	s.c.	none	7.0, 100 days
	178	7.0	s.c.	none	none
	182	7.0	i.m.	none	none
	179	6.0	s.c.	none	none
	183	6.0	i.m.	none	none
	180	5.0	s.c.	none	none
	184	5.0	i.m.	none	none
Chimpanzee	431	7.0	i.m.	none	7.0, 84 days
	457	7.0	s.c.	none	7.0, 84 days
Squirrel monkey	37, 53	6.5	s.c.	5.0, ALVAC-RG	180 days
	38, 54	6.5	s.c.	7.0, ALVAC-RG	180 days
	22, 51	6.5	s.c.	5.0, ALVAC	180 days
	39, 55	6.5	s.c.	5.0, ALVAC-FL[b]	180 days
	57	6.5	s.c.	none	none

[a]Virus dose expressed as $\log_{10}$ p.f.u. per ml
[b]A canarypox recombinant expressing the envelope glycoprotein of FeLV (Tartaglia *et al.*, unpublished data)

such that the flanking arms of the plasmid directed replacement of the non-essential ORF with the foreign gene. Insertion of the foreign gene was accomplished without altering neighboring ORFs and the generation of novel ORFs was precluded by engineering translational stop codons in all appropriate reading frames. The derived recombinant, ALVAC-RG (vCP65), was confirmed to contain the rabies G expression cassette in the correct C5 locus by Southern blot analysis, PCR analysis, and nucleotide sequence analysis (data not presented). Further, expression analyses by immunofluorescence and immunoprecipitation using a rabies G glycoprotein-specific monoclonal antibody confirmed the expression of the 66 kDa rabies glycoprotein on the surface of ALVAC-RG infected cells.

In vitro studies

Analysis of expression of the rabies G gene in avian and human derived cells. Prior results have indicated that ALVAC and derived recombinants do not productively replicate in a range of non-avian cell lines, including those derived from monkey, mouse, cat and human[18] (unpublished results). Additionally, in a similar study to that described in Taylor *et al.* [1] attempts to adapt ALVAC and ALVAC-RG (vCP65) to grow in two non-avian cell lines (MRC-5 and VERO) have failed[25]. Blind passages of both ALVAC and ALVAC-RG were performed in VERO, MRC-5 and primary CEF monolayers for 8 or 10 sequential passages of 7 days duration. While a 100-fold increase in viral titer was apparent in CEF cells after each passage in the series, after one passage in mammalian cells, the viral titer was lower than the residual input titer and titers fell below the level of detectability after two passages[25].

In order to establish that in the absence of productive viral replication the rabies glycoprotein (G) was expressed in the human derived cell lines, immunoprecipitation experiments were performed. The results of a representative analysis are shown in *Figure 1*. No specific immunoprecipitation products were detected in lysates derived from uninfected cells (lanes a, d and g) or cells infected with the parental ALVAC virus (lanes b, e and h). Immunoprecipitation of a 66 kDa protein by the rabies-specific monoclonal antibody was apparent from lysates derived from ALVAC-RG infected CEF, HNK and HEL cells (*Figure 1*, lanes c, f and i, respectively). This size is consistent with that described for SDS–PAGE of the rabies glycoprotein G[26].

In order to determine whether expression of the rabies G gene product would be maintained in human derived cells inoculated with ALVAC-RG (vCP65) in the absence of productive replication, a time course study was performed as described in Materials and Methods. Immunoprecipitation of the rabies G is shown in *Figures* 2a (CEF cells) and 2b (MRC-5 cells). Expression of the rabies G in both CEF and MRC-5 cells occurs as early as 1 h post-infection and continues undiminished under the control of the early/late H6 promoter throughout the labelling period of 24 h.

Analysis of viral specific DNA accumulation in human derived cells inoculated with ALVAC-RG (vCP65). In order to assess the temporal nature of the block in viral replication in human derived cells, the following experiment was performed. Permissive CEF cells and the six human derived cell lines were inoculated with ALVAC parental virus at an MOI of 5 pfu per cell in the presence or absence of AraC, an inhibitor of DNA replication, and the level of virus specific DNA accumulated at 72 h was assessed as described in Materials and Methods. *Figure 3* illustrates analysis of CEF, WISH and DETROIT 532 cells. In the permissive cell line, CEF (*Figure 3*; Panel B), no viral-specific DNA is seen in lane B1 (uninfected CEF cells), lane B2 (ALVAC-infected CEF cells at t_0) or lane B4 ALVAC infected CEF cells at 72 h post-infection in the presence of AraC). Viral specific DNA accumulation represented by a band at approximately 330 kbp is evident in ALVAC infected CEF cells incubated for 72 h in the absence of AraC (lane B3). No such accumulation is seen in the equivalent sample of ALVAC infected DETROIT-532 (lane A3) or WISH (lane C3) cells. Similar results were observed on analysis of ALVAC specific DNA accumulation in MRC-5, HEL, HNK and JT-1 infected cells (results not shown). Based on the conditions employed in these studies, the sensitivity of detection was determined as ≥ 125 genome equivalents. In further experiments, ^{3}H-thymidine incorporation into ALVAC-infected MRC-5 and CEF cells was monitored. These experiments indicated that while an increase in ^{3}H-thymidine incorporation occurred in CEF cells

following infection with ALVAC, in MRC-5 cells, ^{3}H-thymidine incorporation did not rise above basal levels (results not shown). The results indicate that under these conditions, no detectable ALVAC-specific DNA accumulation occurred in the human cell substrates and suggest that replication of ALVAC in these human cells is blocked prior to viral DNA synthesis.

In vivo safety studies with ALVAC-RG (vCP65)

Inoculation of laboratory animals. Previous experiments with CPV and FPV based recombinants in numerous species including mice, rabbits, rats, guinea pigs, cats, dogs, horses, cattle, and swine had demonstrated no adverse reactions upon inoculation by a variety of routes. It was important, however, to examine more stringently the safety profile of an ALVAC-based recombinant in mammalian species.

In a series of experiments described in Materials and Methods and *Table 1*, mice, guinea pigs and rabbits were inoculated with ALVAC-RG by a variety of routes and reactivity monitored. The results indicated that no reactivity was evident in any species following inoculation by the subcutaneous route. Similarly, there was no evidence of neurovirulence apparent in mice or rabbits inoculated with approximately 6.0 $\log_{10}$ $TCID_{50}$ of ALVAC-RG by the intracranial route. Ten rabbits inoculated in this manner showed no local or systemic adverse reactivities, exhibited normal weight gain and no lesions were found in the brain. In mice, in which histopathology was performed, there was no indication of encephalopathy caused by ALVAC-RG (vCP65) in the sections observed. Only very high doses of ALVAC were found to be lethal by i.c. inoculation in young adult or newborn mice further suggesting the lack of neurovirulence of the ALVAC virus[18].

After inoculation by the i.d. route, reactions were evident at the site of inoculation in mice, guinea pigs, and rabbits. In mice, the reactions consisted of small necroses, a few millimeters in diameter, at the site of inoculation. These were evident by 1 day post-

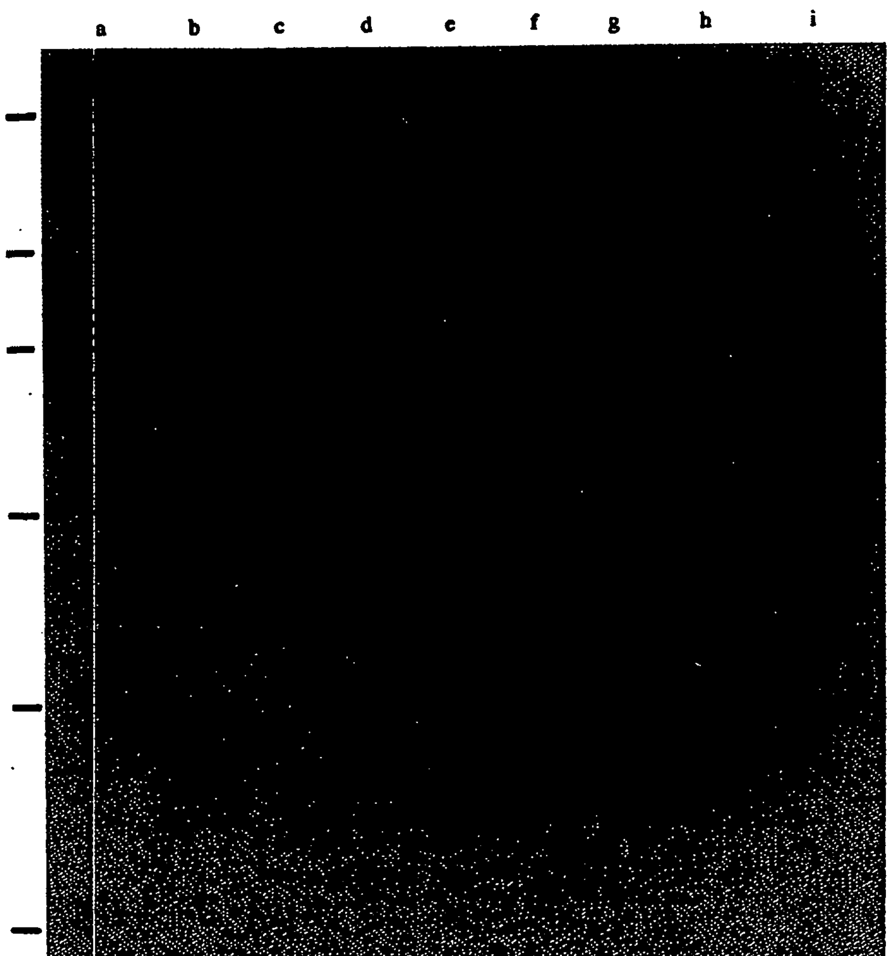


Figure 1 Immunoprecipitation analysis of expression of the rabies glycoprotein in avian and non-avian cells inoculated with ALVAC-RG (vCP65). Dishes of each cell line were inoculated at an input multiplicity of 10 p.f.u./cell with ALVAC or ALVAC-RG in the presence of ^{35}S methionine as described in Ref. 18. Lanes a, d, g, uninfected cells; lanes b, e and h, ALVAC infected cells; lanes c, f and i, ALVAC-RG (vCP65) infected cells. Lanes a, b and c, CEF cells; lanes d, e and f, HNK cells; lanes g, h and i, HEL cells. Molecular weight markers are shown to the left of lane a and indicate migration distances for standard proteins with molecular weights (from the top) of 200, 97.4, 68, 43, 29, 18.4 and 14.3 kDa

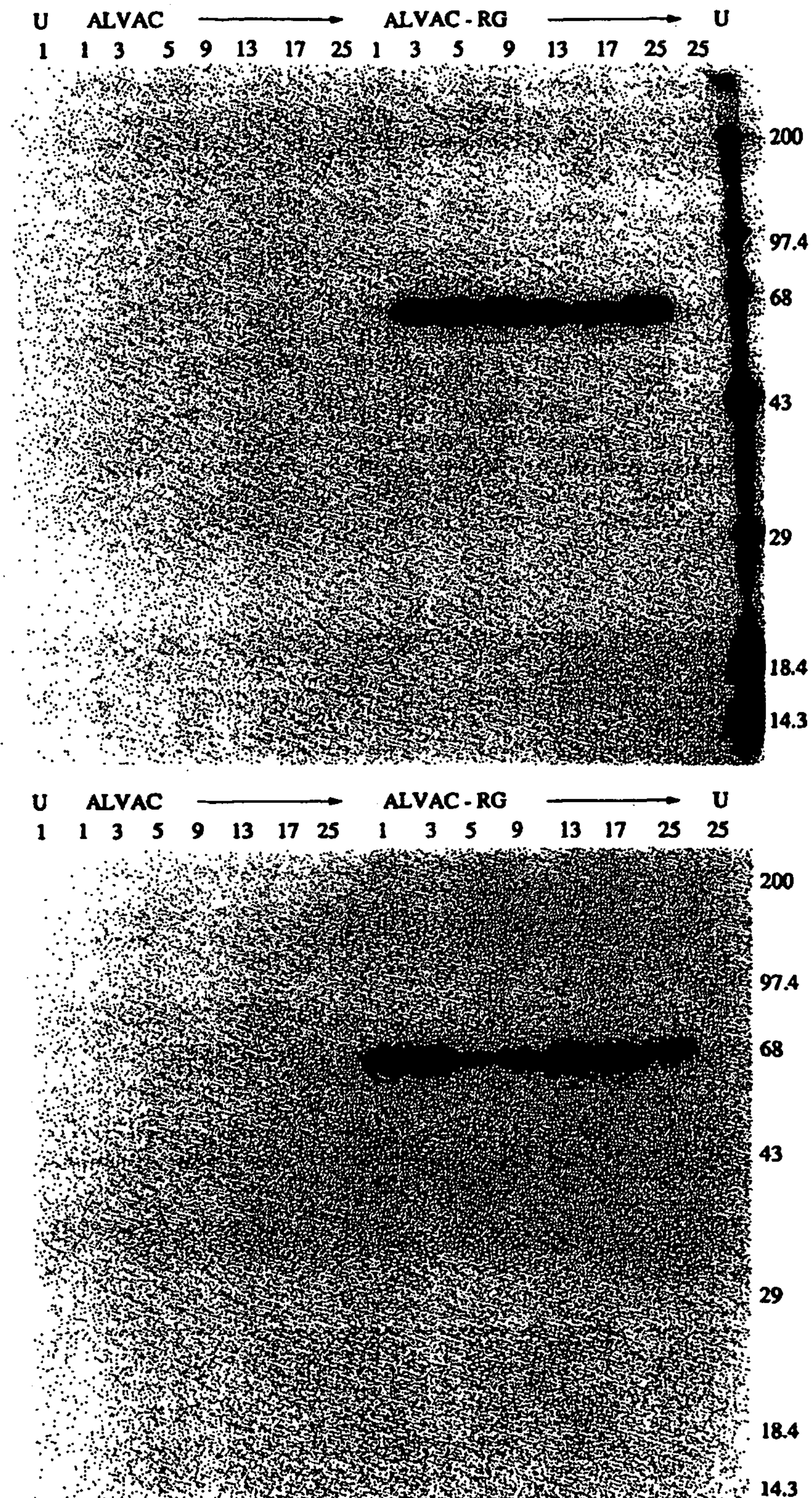


Figure 2 Time course of analysis of expression of the rabies G protein in avian and human-derived cells inoculated with ALVAC-RG. Dishes of CEF or MRC-5 cells were inoculated at an input multiplicity of 10 p.f.u. per cell with ALVAC or ALVAC-RG and labelled for 1 h with ^{35}S-methionine at appropriate times as described in Materials and Methods. Immunoprecipitation was performed as described previously[18]. Figure 2a illustrates analysis of CEF cells and 2b illustrates analysis of MRC-5 cells. U denotes an uninfected control cell lysate, ALVAC or ALVAC-RG indicates cells were infected with ALVAC parental virus or ALVAC-RG, respectively. Figures at the top of each lane indicate time of the labelling period in hours post-infection. Molecular weight markers are shown at the right of each figure (a and b) for migration of standard proteins as described in the legend to Figure 1

inoculation persisting approximately 7 days. In guinea pigs, there was a more inflammatory reaction which consisted of erythema, then a small pustule followed by necrosis. In rabbits, an inflammatory reaction was also seen which progressed to a small pustule with necrotic patches. In both guinea pigs and rabbits the dermal lesions were resolved by 21 days post-inoculation. In a similar experiment, serial dilutions of ALVAC-RG (vCP65) and KANAPOX, the original vaccinal strain of canarypox from which ALVAC was derived, were inoculated by the i.d. route into rabbits and the reactogenicity assessed. Both ALVAC-RG (vCP65) and Kanapox induced some erythema and edema with slight necrosis at the inoculation site indicating the reactogenicity was similar with the parental strain and ALVAC-RG recombinant virus. The reactivity was dose related being most pronounced with the undiluted preparation which contained 6.2 $\log_{10}$ $TCID_{50}$ per ml. It should be noted that viral preparations used in these experiments were not gradient purified and the presence of cellular components may have contributed to the reactogenicity seen following injection by the i.d. route.

Inoculation of canary birds with ALVAC-RG. In order to confirm that the virulence of the parental strain had not been altered by deletion of the C5 ORF and insertion of a heterologous coding sequence (the rabies G gene), reactogenicity of Kanapox and ALVAC-RG (vCP65) was compared in canary birds. No deaths occurred in any of the birds and body weights varied within physiological limits throughout the experimental period. One bird in each group inoculated with 5.0 $\log_{10}$ p.f.u. of ALVAC-RG or Kanapox showed mild inflammation at the application site during the second week post-inoculation with redness and some swelling. All birds inoculated with 7.0 $\log_{10}$ p.f.u. of either virus developed a typical pox-like take on day 5 with inflammation, swelling, a small pock, and in one bird inoculated with ALVAC-RG (vCP65), a patch of

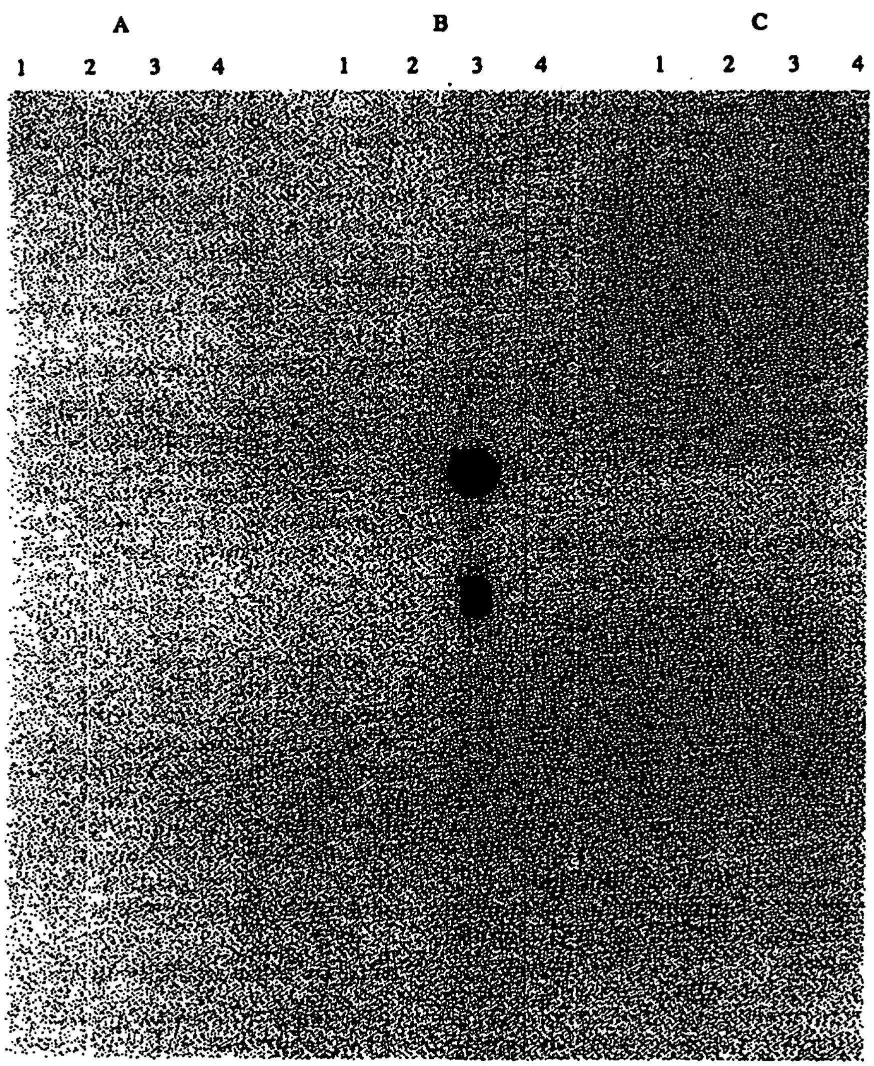


Figure 3 Analyses of viral-specific DNA accumulation in avian and human derived cell lines inoculated with ALVAC. Dishes of each cell line were inoculated and processed as described in Materials and Methods. Panel A, DETROIT 532 cells; Panel B, CEF cells, Panel C, WISH cells. In each panel, lane 1 represents uninoculated cells, lane 2 represents ALVAC-infected cells harvested at zero time, lane 3 represents ALVAC-infected cells harvested at 72 h and lane 4 represents ALVAC-infected cells incubated in the presence of 40 $\mu g\ ml^{-1}$ of AraC and harvested at 72 h

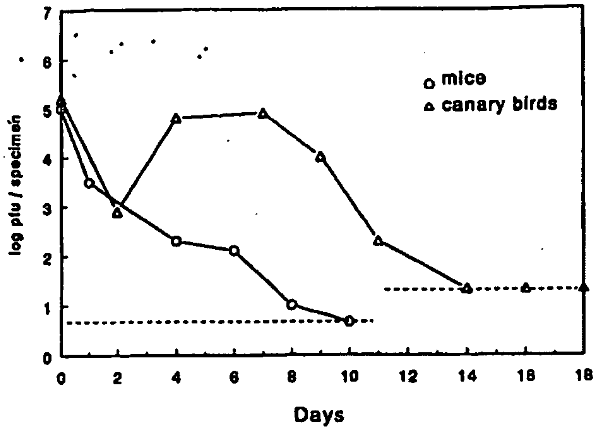


Figure 4 Analysis of virus isolation from skin of canaries and mice inoculated with ALVAC-RG. Mice and canaries were inoculated with ALVAC-RG by the i.d. route as described in Materials and Methods. At time intervals after inoculation animals and birds were sacrificed and the skin surrounding the site of inoculation was dissected, homogenized and assayed for the presence of infectious virus by titration on QT35 cells

necrosis. All lesions remained localized and were resolved by 21 days post-inoculation.

A further experiment was performed to follow the fate of inoculated virus in canary birds and in mice. Mice were inoculated by the i.d. route in the ear pinna and canary birds by smearing virus onto an area of skin on the back of the bird from which feathers had been plucked. The results of virus isolation from the area of inoculation are shown in *Figure 4*. The results indicate that in canary birds, there was an initial eclipse phase of 2 days after which an increase in infectious virus was observed for up to 7 days. Viral recovery then declined and after 14 days no virus was detected. In contrast, from day 0 the amount of virus recovered from mice progressively declined until reaching the limit of detection (20 p.f.u. per sample) on day 10 after inoculation.

In vivo studies: inoculation of non-human primates

Safety and immunogenicity in squirrel monkeys. Three groups of four squirrel monkeys (*Saimiri sciureus*) were inoculated as described in MATERIALS AND METHODS and *Table 2*, and monitored for reactogenicity as well as immune response. Animals received either ALVAC parental virus (animal Nos 22, 51, 36, and 52), ALVAC-RG (vCP65 animal) (Nos 37, 53, 38, and 54) or a canarypox recombinant expressing an Env gene product derived from an endogenous FeLV provirus (animal Nos 39, 55, 40, and 56). The initial inoculation was performed by ocular, oral and i.d. routes. No reactions were seen following inoculation of the three viruses except for minor skin lesions following i.d. inoculation of approximately 7.0 $\log_{10}$ p.f.u. Both body weight and temperature of all animals remained within normal limits. Virus isolation from ocular fluid, saliva and the site of i.d. inoculation was attempted for 11 days post-inoculation. Virus recovery was achievable from the inoculation site for 2 (6/6 animals) to 4 days (2/6 animals) following the i.d. administration of 7.0 $\log_{10}$ p.f.u. but not 5.0 $\log_{10}$ p.f.u. of all viruses. Virus was not recoverable from eye secretions or saliva at any timepoint (results not shown).

Analysis of post-inoculation sera by ELISA indicated that all animals inoculated with either ALVAC or an ALVAC-based recombinant developed a serological response to ALVAC (results not shown). All four animals inoculated with ALVAC-RG (vCP65) (animal Nos 37, 53, 38, and 54) developed rabies virus neutralizing antibody (*Table 3*), the level of which at 28 days was well above that considered to be a satisfactory response to rabies vaccination. It should be noted that 0.5 International Units, or a titer of approximately 1:16 is considered by the WHO to be the acceptable minimal response to rabies vaccination[27]. Six months after the primary inoculation, four monkeys which received ALVAC-RG (vCP65) (37, 53, 38, and 54), two monkeys which received ALVAC (22, 51), two monkeys which received an ALVAC recombinant expressing the FeLV env gene (39, 55), and one naive monkey (57) were inoculated with 6.5 $\log_{10}$ p.f.u. of ALVAC-RG (vCP65) by the s.c. route to monitor the immune response in the face of pre-existing ALVAC immunity. There were no adverse reactions to re-inoculation in any of the animals. At 28 weeks all previously inoculated animals showed some low level of canarypox ELISA antibody which was boosted 3–7 days after reinoculation (results not shown). Assessment of levels of anti-rabies antibody in sera of these animals is shown in *Table 3*. The four animals with prior exposure to ALVAC (22 and 51) or ALVAC-FeLV (39 and 55) and the naive animal (57) mounted a primary response with rabies virus neutralizing antibody present 7–11 days post-inoculation. Significantly, the four monkeys with prior exposure to ALVAC-RG (vCP65) showed an anamnestic response by 7 days post-inoculation.

Safety and immunogenicity in Rhesus macaques

Two macaques were initially inoculated with ALVAC-RG as described in *Table 2* by the s.c. route. No local or systemic adverse reactions to inoculation were noted. After 100 days, these animals were reinoculated by the s.c. route and an additional six animals were inoculated with a range of doses by the i.m. or s.c. routes. Sera of animals were monitored for the presence of anti-rabies neutralizing antibody in the RFFIT-test[24] and results are shown in *Table 4*. Animals 177 and 186 receiving ALVAC-RG (vCP65) by the s.c. route developed rabies virus neutralizing antibody detectable at 11 days post primary inoculation. Levels of antibody above the minimal acceptable level[27] were still present at 3 months when animals were re-inoculated and both animals responded with an increase in titer. Equivalent responses were obtained by either the s.c. or i.m. routes with a dose of either 7.0 or 6.0 $\log_{10}$ p.f.u. At a dose of 5.0 $\log_{10}$ p.f.u. only one animal (180) responded by the s.c. route.

Safety and immunogenicity studies in chimpanzees

Two chimpanzees were inoculated by the i.m. (animal 431) or s.c. (animal 457) routes with 7.0 $\log_{10}$ p.f.u. of ALVAC-RG (vCP65). At 12 weeks, both animals were re-inoculated in an identical manner. No local or systemic adverse reactions to inoculation were noted in either animal. Serological results are shown in *Table 5*. Both chimpanzees responded with the induction of

Table 3 Response of squirrel monkeys with prior exposure to ALVAC or ALVAC-based recombinants to inoculation with ALVAC-RG

Animal number	First inoc.	RFFIT titer at day post-inoculation[a] 0	7	28	180[d]	187	201
22	ALVAC	—	—	—	<16	<16	200
51	ALVAC	—	—	—	<16	50	158
39	ALVAC-FL	—	—	—	<16	50	158
55	ALVAC-FL	—	—	—	<16	50	126
37[b]	ALVAC-RG	<16	<16	1000	<16	1580	3160
53[b]	ALVAC-RG	<16	16	158	<16	3980	3980
38[c]	ALVAC-RG	<16	316	1000	<50	1580	3980
54[c]	ALVAC-RG	<16	250	1580	<50	3980	10000
57	None	—	—	—	<16	50	500

[a]Sera tested in an RFFI Test (Ref. 24). Titer expressed as eciprocal of the highest dilution showing complete inhibition of fluorescence.
[b]Animals 37 and 53 were inoculated with 5.0 $\log_{10}$ p.f.u. as described in Table 2
[c]Animals 38 and 54 were inoculated with 7.0 $\log_{10}$ p.f.u. as described in Table 2
[d]At day 180, all animals were inoculated with 6.5 $\log_{10}$ p.f.u. of ALVAC-RG by the subcutaneous route

rabies virus neutralizing antibody at 2–4 weeks post-inoculation and antibody titers were significantly boosted after the second inoculation at 12 weeks.

DISCUSSION

The studies described in this communication were conceived to evaluate, in some detail, the biological and immunological properties of ALVAC and derivative recombinants in non-avian species. The results provide a safety profile for the ALVAC vaccine vector and illustrate the utility of ALVAC as a general immunization vehicle in non-avian species.

Failure to demonstrate replication of ALVAC or ALVAC-based recombinants has been demonstrated on tissue culture cells of murine and feline origin (unpublished data). Further, no evidence for viral replication has been obtained following inoculation of ALVAC on a variety of human or monkey-derived tissue culture systems[18] and the inability to adapt the virus to growth on human or monkey-derived cell lines has been confirmed by serial blind passage of both ALVAC and ALVAC-RG (vCP65)[25].

Table 4 Serological response following inoculation of Rhesus macaques with ALVAC-RG

Animal No. /dose	Route 1°/2°	RFFIT titer at days post-inoculation 0	6	11	35	99[a]	101	105	114	128
177/7.7	s.c./s.c.[b]	—	—	16[d]	32	64	32	512	512	256
186/7.7	s.c./s.c.	—	—	128	512	256	256	512	512	256
178/7.0	s.c.					—	—	—	64	64
182/7.0	i.m.[c]					—	—	—	32	64
179/6.0	s.c.					—	—	—	64	32
183/6.0	i.m.					—	—	—	128	128
180/5.0	s.c.					—	—	—	32	32
184/5.0	i.m.					—	—	—	—	—

[a]Day of re-inoculation
[b]Subcutaneous route
[c]Intramuscular route
[d]Titers expressed as reciprocal of last dilution showing inhibition of fluorescence in RFFI test[24]

Table 5 Serological response of chimpanzees to inoculation with ALVAC-RG

Animal No. /route	RFFIT titer at weeks post-inoculation 0	1	2	4	8	12[a]	13	15	20	26
431/i.m.	<8[b]	<8	8	16	16	16	128	256	64	32
457/s.c.	<8	<8	32	32	32	8	128	512	128	128

Animals were inoculated with 7.0 $\log_{10}$ p.f.u. of ALVAC-RG by the indicated route and re-inoculated in the same manner 12 weeks later
[a]Time of reinoculation
[b]Titer expressed as reciprocal of last dilution showing inhibition of fluorescence in an RFFI test[24]

On human-derived cell cultures, no accumulation of ALVAC-specific viral DNA was demonstrated suggesting that the block to viral replication in these cell substrates occurs early in the replication cycle prior to viral DNA replication. Similar analyses performed with ALVAC-infected VERO cells have demonstrated low, but detectable, levels of accumulated ALVAC-specific DNA (data not shown). Somogyi *et al.*[28] have recently shown that in MRC-5 cells infected with fowlpox virus, both viral DNA replication and some late viral protein synthesis can be detected, albeit at reduced levels. The block in avipox productive replication in mammalian cells may vary, not only for different cell types, but also for the different avipox viruses. While the details of the molecular events responsible for the block to viral replication in non-avian species remain to be elucidated, it is significant that the expression of at least some avipox virus genes, and of appropriately regulated extrinsic immunogens, occurs in all non-avian tissue cultures tested. Additionally, when the time course of expression of the rabies G gene was monitored in these cells, it was evident that expression could be detected continuously from 1 to 25 h post-infection when the experiment was terminated (*Figure 2*).

Previous *in vivo* studies in a variety of species including mice, cats, and dogs[2,3] had shown no reactogenicity following inoculation of a CPV recombinant. A number of laboratory animals were inoculated with ALVAC or ALVAC-RG (vCP65) to extend these results. Safety studies performed in laboratory animals via the s.c. and i.m. routes indicated no reactogenicity. Similarly, inoculation of mice and rabbits by the i.c. route showed no evidence of neurovirulence. This is also supported by data of LD_{50} values by i.c. inoculation of young or newborn mice[18]. Further, no adverse reactions have been observed upon inoculation of immunodeficient mice[18].

Inoculation of rabbits with high doses of CPV by the i.d. route resulted in the formation of poxvirus-like lesions. In related experiments not reported here, lesions were induced on rabbits by i.d. inoculation of 8.0 $\log_{10}$ p.f.u. of ALVAC. When the dose was reduced to 7.0 and 6.0 $\log_{10}$ p.f.u., minimal reactogenicity was apparent. Similarly, skin lesions were evident at the site of i.d. inoculation of ALVAC and derived recombinants in squirrel monkeys but only sporadic virus recovery was possible through 4 days post inoculation. The formation of a lesion at the site of i.d. inoculation may be a cytotoxic phenomenon due to expression of early viral functions or may be linked to the presence of cellular components in the inoculum. Reactogenicity by the i.d. route was not related to altered pathogenicity following insertion of a foreign gene since an equivalent effect was seen with the parental canarypox vaccine strain, Kanapox.

Three non-human primate species were inoculated with ALVAC-RG (vCP65) to monitor safety and immunogenicity. No adverse signs of infection or disease were seen in the squirrel monkeys, macaques, or chimpanzees following inoculation by a variety of routes. All three species responded with significant levels of rabies virus neutralizing antibody which were boosted after a second inoculation. Significantly, squirrel monkeys with a history of prior exposure to CPV or CPV recombinants did not show a diminution of response when inoculated with ALVAC-RG (vCP65). These monkeys have since been inoculated with a third ALVAC recombinant expressing the measles virus fusion and hemagglutinin glycoproteins and have responded with protective levels of measles virus HI antibody comparable to that induced in naive animals (unpublished data). These results indicate that prior exposure to ALVAC recombinants should not preclude subsequent vaccinations with a novel ALVAC recombinant. In addition, it should be noted that four of the monkeys (22, 37, 38 and 39) had also received vaccinia virus three months before inoculation with ALVAC or ALVAC-based recombinants. The fact that the rabies-specific immune response was not diminished in these animals may indicate that in humans, prior immunity to vaccinia virus may not limit use of an ALVAC-based recombinant vaccine. The concept of using a non-replicating vector system in humans has been demonstrated in Phase 1 clinical trials with the ALVAC-RG (vCP65) recombinant virus. Volunteers inoculated with ALVAC-RG (vCP65) demonstrated significant immune responses to the extrinsic immunogen in the absence of unacceptable local or systemic reactions to vaccination[10,11].

Practical issues of utilizing ALVAC-based recombinants for specific veterinary applications have been addressed in the target species. In a duration of immunity study, dogs inoculated with a single dose (6.7 $\log_{10}$ $TCID_{50}$) of ALVAC-RG (vCP65) were protected against a lethal challenge with rabies virus at 36 months post-inoculation (manuscript in preparation). Other unpublished studies have provided evidence that these vector systems may be useful in the presence of maternally derived antibodies (manuscript in preparation). The safety and immunogenicity profile of ALVAC-based recombinants suggests a strong potential for ALVAC as a generic immunization vehicle in other veterinary as well as human applications.

ACKNOWLEDGEMENTS

We wish to thank Dr E. Kauffman who performed the fluorescence analysis, Dr G. Johnson who performed the time course studies, and M. Mellon and T. Moran for expert technical assistance. Dr E. Muchmore of the Laboratory of Experimental Medicine and Surgery in Primates, Tuxedo, New York conducted the experimental vaccination of chimpanzees in collaboration with Dr Marc Girard, Institut Pasteur, Paris. T.M. Jourdier performed the studies in canaries, mice and squirrel monkeys. Drs J.C. Moulin and C. Goldman performed the safety studies in rodents. We are grateful to L. Switzer, K. Dombrowski and E. Christiansen who prepared the manuscript. Transgene (Strasbourg, France) provided the cloned rabies glycoprotein gene. Animal procedures have been approved by the Institutional Animal Care and Use Committee.

REFERENCES

1 Taylor, J., Weinberg, R., Languet, B., Desmettre, P. and Paoletti, E. A recombinant fowlpox virus inducing protective immunity in non-avian species. *Vaccine* 1988, **6**, 497–503

2 Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, P. and Paoletti, E. Efficacy studies on a canarypox-rabies recombinant virus. *Vaccine* 1991, **9**, 190–193

3 Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D. *et al.* Nonreplicating viral vectors as potential vaccines: Recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. *Virology* 1992, **187**, 321–328
4 Taylor, J., Tartaglia, J., Moran, T., Webster, R.G., Bouquet, J.F., Quimby, F.W. *et al.* The role of poxvirus vectors in influenza vaccine development. In: *Proceedings of the Third International Symposium on Avian Influenza*, 27–29 May 1992, University of Wisconsin, Madison, WI. 1992, pp. 311–335
5 Wild, F., Giraudon, P., Spehner, D., Drillien, R. and Lecocq, J.P. Fowlpox virus recombinant encoding the measles virus fusion protein: protection of mice against fatal measles encephalitis. *Vaccine* 1990, **8**, 441–442
6 Esposito, J.J. Poxviridae. In: *Classification and Nomenclature of Viruses.* Fifth Report of the International Committee on Taxonomy of Viruses (Eds Francki, R.I.B., Faguet, C.M., Knudson, D. and Brown, F.). *Arch. Virol.* 1991, Supplement 2, Springer, New York, pp. 91–102
7 Tartaglia, J., Jarrett, O., Neil, J.C., Desmettre, P. and Paoletti, E. Protection of cats against Feline Leukemia Virus by vaccination with a canarypox virus recombinant, ALVAC-FL. *J. Virol.* 1993, **67**, 2370–2375
8 Konishi, E., Pincus, S., Paoletti, E., Shope, R.E. and Mason, P.W. Avipox virus-vectored Japanese encephalitis virus vaccines: use as vaccine candidates in combination with purified subunit immunogens. *Vaccine* 1994, **12**, 633–638
9 Cox, W.I., Tartaglia, J. and Paoletti, E. Induction of cytotoxic T lymphocytes by recombinant canarypox (ALVAC) and attenuated vaccinia (NYVAC) viruses expressing the HIV-1 envelope glycoprotein. *Virology* 1993, **195**, 845–850
10 Cadoz, M., Strady, A., Meignier, B., Taylor, J., Tartaglia, J., Paoletti, E. and Plotkin, S. Immunization with canarypox virus expressing rabies glycoprotein. *Lancet* 1992, **339**, 1429–1432
11 Fries, L.F., Tartaglia, J., Taylor, J., Kauffman, E.K., Meignier, B. and Paoletti, E. Human safety and immunogenicity of a canarypox–rabies glycoprotein recombinant vaccine: an alternative pox virus vector system. Submitted for publication
12 Moscovici, C., Moscovici, M.G., Jimenez, H., Lai, M.M.C., Hayman, M.J. and Vogt, P.K. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. *Cell* 1977, **11**, 95–103
13 Karber, G. Beitrag zur Kollektiven Behaudlungpharmakologischer Reihenversuche. *Arch. Exp. Pathol. Pharmakol.* 1931, **162**, 480–483
14 Rickinson, A.B., Rowe, M., Hart, I.J., Yao, Q.Y., Henderson, L.E., Rabin, H. and Epstein, M.A. T-cell mediated regression of "spontaneous" and of Epstein-Barr virus-induced B-cell transformation *in vitro*: studies with cyclosporin A. *Cell Immunol.* 1984, **87**, 646–658
15 Piccini, A., Perkus, M.E. and Paoletti, E. Vaccinia virus as an expression vector. *Meth. Enzym.* 1987, **153**, 545–563
16 Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R.G. and Paoletti, E. Protective immunity against avian influenza induced by a fowlpox virus recombinant. *Vaccine* 1988, **6**, 504–508
17 Maniatis, T., Fritsch, E.F. and Sambrook, J. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982
18 Tartaglia, J., Perkus, M.E., Taylor, J., Norton, E.K., Audonnet, J.-C., Cox, W.I. *et al.* NYVAC: a highly attenuated strain of vaccinia virus. *Virology* 1992, **188**, 217–232
19 Yeun, L. and Moss, B. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. *Proc. Natl Acad. Sci. USA* 1987, **84**, 6417–6421
20 Anilionis, A., Wunner, W.H. and Curtis, P.J. Structure of the glycoprotein gene in rabies virus. *Nature* 1981, **294**, 275–278
21 Kieny, M.P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D. *et al.* Expression of rabies virus glycoprotein from a recombinant vaccinia virus. *Nature* 1984, **312**, 163–166
22 Perkus, M.E., Limbach, K. and Paoletti, E. Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. *J. Virol.* 1989, **63**, 3829–3836
23 Taylor, J., Edbauer, C., Rey-Senelonge, A., Bouquet, J.F., Norton, E., Goebel, S. *et al.* Newcastle disease virus fusion protein expressed in a fowlpox virus recombinant confers protection in chickens. *J. Virol.* 1990, **64**, 1441–1450
24 Smith, J.S., Yager, P.A. and Baer, G.M. A rapid tissue culture test for determining rabies neutralizing antibody. In: *Laboratory Techniques in Rabies* (Eds Kaplan, M.M. and Koprowski, H.). WHO, Geneva, 1973 pp. 354–357
25 Perkus, M.E., Taylor, J., Tartaglia, J., Pincus, S., Kauffman, E. and Paoletti, E. Live attenuated vaccinia and other poxviruses as delivery systems: public health issues. *Ann. New York Acad. Sci.* 1994, in press
26 Dietzschold, B., Wiktor, T.J., Wunner, W.H. and Varrichio, A. Chemical and immunological analysis of the rabies soluble glycoprotein. *Virology* 1983, **124**, 330–337
27 WHO Expert Committee on Rabies. 8th Report. WHO Technical Report Series, No. 824, 1992, Geneva p.22
28 Somogyi, P., Frazier, J. and Skinner, M.A. Fowlpox virus host range restriction: gene expression, DNA replication and morphogenesis in nonpermissive mammalian cells. *Virology* 1993, **197**, 439–444

US005766598A

United States Patent [19]

Paoletti et al.

[11] **Patent Number: 5,766,598**

[45] **Date of Patent: Jun. 16, 1998**

[54] **RECOMBINANT ATTENUATED ALVAC CANARYPOXVIRUS EXPRESSION VECTORS CONTAINING HETEROLOGOUS DNA SEGMENTS ENCODING LENTIVIRAL GENE PRODUCTS**

[75] Inventors: **Enzo Paoletti**, Delmar; **James Tartaglia**, Schenectady; **William Irvin Cox**, Troy, all of N.Y.

[73] Assignee: **Virogenetics Corporation**, Troy, N.Y.

[21] Appl. No.: **303,275**

[22] Filed: **Sep. 7, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 897,382, Jun. 11, 1992, abandoned, which is a continuation-in-part of Ser. No. 715,921, Jun. 14, 1991, abandoned, and Ser. No. 847,951, Mar. 6, 1992, which is a continuation-in-part of Ser. No. 713,967, Jun. 11, 1991, which is a continuation-in-part of Ser. No. 666,056, Mar. 7, 1991.

[51] **Int. Cl.**[6] **A61K 39/12**; A61K 39/295; A61K 39/21; A61K 39/275

[52] **U.S. Cl.** **424/199.1**; 424/208.1; 424/232.1; 424/201.1; 435/320.1; 435/236; 435/172.3

[58] **Field of Search** 435/69.1, 172.3, 435/235, 236, 237; 424/199.1, 202.1, 208.1, 209.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,093,258	3/1992	Cohen	435/235.1
5,174,993	12/1992	Paoletti	424/89
5,180,675	1/1993	Prillien	435/235.1
5,185,146	2/1993	Altenburger	424/89

FOREIGN PATENT DOCUMENTS

WO 89/03429	of 1989	WIPO .
WO A 89/03429	4/1989	WIPO .
WO A 90/10693	9/1990	WIPO .
WO A 90/12101	10/1990	WIPO .

OTHER PUBLICATIONS

Turner, P.C. et al. (90) Current topics in Microbiol. Immunol. 163: 125–151.

Lerner, R.A. et al. (83) in: The Biology of Immunologic Disease, eds. F.J. Dixon & D.W. Fisher, H.P. Publishing Co. New York, NY, pp. 331–338.

Wain-Hobson, S. et al. (85) Cell 40:9–17.

Cooney, E.L. et al. (91) Lancet 337:567–572.

Tartaglia, J. et al. (906) in: Immunochemistry of Viruses, eds. M.H.V. van Regenwortel and A.R. Newrath, Elsevier Science Publishers B.V. pp. 125–151.

Norley, S. et al. Immunobiology 184: 193–207 (1992).

Cohen, J. et al. Science 262: 980–81 (1993).

Moss, B., 1996, "Poxviridae: The Viruses and Their Replication" in *Fields Virology*, Third Edition, Fields et al., eds., Lippincott–Raven Publishers, Philadelphia, pp. 2637–2671.

Murphy, F., 1996, "Virus Taxonomy", in *Fields Virology*, Third Edition, Fields et al., eds., Lippincott–Raven Publishers, Philadelphia, pp. 15–57.

Taylor et al., 1991, Vaccine 9(3):190–193.

Berman et al., 1990, Nature 345:622–625.

Girard et al., 1991, Proc. Natl. Acad. Sci. USA 88:542–546.

Hu et al., 1986, Nature 320:537–540.

Ho et al., 1990, Eur. J. Immunol. 20:477–483.

Virology, vol. 173, No. 1, issued Nov. 1989, S. Dallo et al., "Humoral Immune Response Elicited by Highly Attenuated Variants of Vaccinia Virus and by an Attenuated Recombinant Expressing HIV-1 Envelope Protein", pp. 323–328 (entire document).

Virology, vol. 152, issued 1986, M.E. Perkus et al., "Insertion and Deletion Mutants of Vaccinia Virus", pp. 285–297 (entire document).

Nature, vol. 317, issued 31 Oct. 1985, R.M.L. Buller et al., "Decreased Virulence of Recombinant Vaccinia Virus Expression Vectors is Associated With a Thymidine Kinase-Negative Phenotype", pp. 813–815 (entire document).

Journal of Virology, vol. 62, No. 12, issued Dec. 1988, H. Shida et al., "Effects and Virulences of Recombinant Vaccinia Viruses Derived From Attenuated Strains That Express the Human T-Cell Leukemia Virus Type 1 Envelope Gene", pp. 4474–4480 (entire document).

Critical Reviews in Immunology, vol. 10, No. 1, issued 1990, J. Tartaglia et al., "Poxvirus-Based Vectors as Vaccine Candidates", pp. 13–30 (entire document). (Tartaglia 900).

Virology, vol. 188, No. 1, issued May 1992, J. Tartaglia et al., "NYVAC: A Highly Attenuated Strain of Vaccinia Virus", pp. 217–232 (entire document).

Hosmalin, A., Nara, P.L., Zweig, M., Lerche, N.W., Cease, K.B., Gard, E.A., Markham, P.D., Putney, S.D., Daniel, M.D., Desrosiers, R.C., and Berzofsky, J.A., J. Immunol. 146, 1667–1673 (1991).

(List continued on next page.)

Primary Examiner—Laurie Scheiner
Assistant Examiner—Jeffrey S. Parkin
Attorney, Agent, or Firm—Frommer Lawrence & Haug LLP; William S. Frommer; Thomas J. Kowalski

[57] **ABSTRACT**

This invention is directed toward recombinant attenuated canarypox virus expression vectors containing exogenous DNA segments encoding lentiviral gene products. A parental canarypox virus (Rentschler strain) was obtained and attenuated through more than 200 serial passages on chick embryo fibroblasts. A master viral seed was subjected to four successive plaque purifications under agar and one plaque clone was amplified through five additional passages after which the stock virus was used as the parental virus in in vitro recombination studies. This attenuated plaque purified canarypox isolate was designated ALVAC. A series of ALVAC recombinants were generated that are capable of expressing different HIV and SIV gene products including Gag, Pol, Env, and Nef. These recombinants provide useful reagents for the generation of viral-specific immune responses.

14 Claims, 9 Drawing Sheets

OTHER PUBLICATIONS

Letvin, N.L., and King, N.W., J. AIDS 3, 1023–1040 (1990).
McMichael, A.J., Gotch, F.M., Noble, G.R., and Beare, P.A.S., New Engl. J. Med. 309, 13–17 (1983).
Moss, D.J., Rickinson, A.B., and Pope, J.H., Int. J. Cancer 22, 662–668 (1978).
Schmaljohn et al., Virology 155, 633–643 (1986).
Schmitt et al., J. Virol. 62, 1889–1897 (1988).
Tartaglia, J., Winslow, J., Goebel, S., Johnson, G.P., Taylor, J., and Paoletti, E., J. Gen. Virol. 71, 1517–1524 (1990).
Tartaglia et al., Virology 188, 217–232 (1992).
Taylor et al., Virology 187, 321–328 (1992).
Vos et al., EMBO J. 7, 3487–3492 (1988).
Walker, B.D. and Plata, F., AIDS 4, 177–184 (1990).
Walker, B.D., Chakrabarti, S., Moss, B., Paradi, T.J., Flynn, T., Durno, A.G., Blumberg, R.S., Kaplan, J.C., Hirsch, M.S., and Schooley, R.T., Nature 328, 345–348 (1987).
Walker, B.D., Flexner, C., Birch–Limberger, K., Fisher, L., Paradis, T.J., Aldovini, A., Young, R., Moss, B., and Schooley, R.T., Proc. Natl. Acad. Sci. 86, 9514–9519 (1989).
Weiss, R.A., Clapham, P.R., Cheingsong–Popov, R., Dalgleish, G., Carne, C.A., Weller, I.V., and Tedder, R.S., Nature 316, 69–72 (1985).
Yuen, L., and Moss, B., Proc. Natl. Acad. Sci. USA 84, 6417–6421 (1987).
Zagury, D., Bernard, J., Cheynier, R., Desportes, I., Leonard, R., Fouchard, L. Reveil, B., Ittele, D., Lurhuma, Z., Mbayo, K., Wane, J., Salaun, J.–J., Goussard, B., Dechazal, L., Burny, A., Nara, P. and Gallo, R.C., Nature 332, 728–731 (1988).
Zarling, J.M., Morton, W., Moran, P.A., McClure, J., Kosowski, S.G., and Hu, S.–L., Nature 323, 344–346 (1986).
Autran, B., Plata, F., and Debre, P., J. AIDS 4, 361–367 (1991).
Borysiewicz, L. Graham, S., Hickling, J., Mason, P.D., and Sissons, J.P.G., Eur. J. Immunology 18. 269–275 (1988).
Good, M.F., Maloy, W.L., Lunde, M.N., Margalit, H., Cornette, J.L., Smith, G.L., Moss, B., Miller, L.H., and Berzofsky, J.A., Science 235, 1059–1062 (1987).
Guo, P., Goebel, S., Davis, S., Perkus, M.E., Taylor, J., Norton, E., Allen, G., Lanquet, B., Desmettre, P., and Paoletti, E., J. virol. 174, 217–214 (1990).
Ho, P.C., Mutch, D.A., Winkel, K.D., Saul, A.J., Jones, G.L., Doran, T.J., and Rzepczyk, C.M., Eur. J. Immunol. 20, 477–483 (1990).
Ruegg, C.L., Monell, C.R., and Strand, M., J. Virol. 63, 3257–3260 (1989b).
Sanchez–Pascador, R., Power, M.D., Barr, P.J., Steimer, K.S., Stampien, M.M., Brown–Shimer, S.L., Gee, W., Renard, A., RAndolph, A., Levy, J.A., Dina, D., and Luciw, P.A., Science 227, 484–492 (1985).
Sanger, F., Nickeln, S., and Coulson, A. R., Proc. Natl. Acad. Sci. 74, 5463–5467 (1977).
Shaffermann, A., Lennox, J., Grosfeld, H., Sadoff, J., Redfield, R.R., and Burke, D.S., AIDS Research and Human Retroviruses 5, 33–39 (1989).
Shapira, S.K., Chou, J., Richaud, F.V., and Casadaban, M.J., Gene 25, 71–82 (1983).
Shioda, T. and H. Shibuta, Virology 175, 139–148 (1990).
Starcich et al., Cell 45, 637–648 (1986).
Tabor, S., and Richardson, C.C., Proc. Natl. Acad. Sci. USA 84, 4767–4771 (1987).
Tartaglia, J. and Paoletti, E., In Immunochemistry of viruses, II. eds. van Regenmortel, M.H.V and Neurath, A.R., (Elsevier Science Publishers B.V., Amsterdam) pp. 125 (1990).
Taylor, J. et al., Vaccine 6, 466–467 (1988).
Taylor, J. et al., Vaccine 9, 190–193 (1991).
Taylor, J., Weinberg, R., Lanquet, B., Desmettre, P., and Paoletti, E., Vaccine 6, 497–503 (1988a).
Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R.G., and Paoletti, E., Vaccine 6, 504–508 (1988b).
Walker, B.D., Flexner, C., Paradis, T.J., Fuller, T.C., Hirsch, M.S., Schooley, R.T. and Moss, B., Science 240, 64–66 (1988).
Nixon, D.F., Townsend, A.R.M., Elvin, J.G., Rizza, C.R., Gallwey, J., and McMichael, A.J., Nature 326, 484–487 (1988).
Panicali, D., and Paoletti, E., Proc. Natl. Acad. Sci. USA 79, 4927–4931 (1982).
Perkus, M.E., Piccini, A., Lipinskas, B.R., and Paoletti, E., Science 229, 981–984 (1985).
Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K., and Paoletti, E., Virology 179, 276–286 (1990).
Perkus, M.E., Limbach K., and Paoletti, E., J. Virol. 63, 3829–3836 (1989).
Piccini, A., Perkus, M.E., and Paoletti, E., Methods in Enzymology 153, 545–563 (1987).
Plata, F., Autran, B., Martins, L.P., Wain–Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J.–M., Debre, P., Nature 328, 348–351 (1987).
Ratner, L., Hasseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, Jr., S.R., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo., R.C., and Wong–Staal F., Nature 313, 277–284 (1985).
Rautmann, G., Kieny, M.–P., Brandely, R., Dott, K., Girard, M. Montagnier, L., and Lecocg, J.–P. AIDS Research and Human Retroviruses 5, 147–157 (1989).
Riviere Y., Tanneau–Salvadori, F., Regnault, A., Lopez, O., Sansonetti, P., Guy, B., Kieny, M.–P., Fournel, J.–J., and Montagnier, L., J. Virol. 63, 2270–2277 (1989).
Rodriguez, D. et al., Proc. Natl. Acad. Sci. USA 86, 1287–1291 (1989).
Ruegg, C.L., Monell, C.R., and Strand, M., J. Virol. 63, 3250–3256 (1989a).
Karacostas, V., Nagashima, K., Gonda, M.A., and Moss, B., Proc. Natl. Acad. Sci. USA 86, 8964–8968 (1989).
Klasse, P.J., Pipkorn, R., and Blomberg, J., Proc. Natl. Acad. Sci. USA 85, 5225–5229 (1988).
Kodama, T., Wooley, D.P., Naidu, Y.M., Kestler III, H.W., Daniel, M.D., Li, Y., and Derisiers, R.C., J. Virol. 63, 4709–4714 (1989).
Koff, W.C. and Fauci, A.S., AIDS 1, S125–S129 (1989).
Koup, R.A., Sullivan, J.L., Levine, P.H., Brettler, D., Mahr, A., Mazzara, G., McKenzie, S., and Panicali, D., Blood 73, 1909–1914 (1989).
Kunkel, T.A., Proc. Natl. Acad. Sci. USA 82, 488–492 (1985).
Laemmli, U.K., Nature (London) 227, 680–685 (1970).
Lane, J.M., Ruben, F.L., Neff, J.M., and Millar, J.D., New Eng. J. Med. 281 1201–1208 (1969).

LaRosa, G.J., Davide, J.P., Weinhold, K., Waterbury, J.A., Profy, A.T., Lewis, J.A., Langlois, A.J., Dressman, G.R., Boswell, R.N., Shadduck, P., Holley, L.H., Karpus, M., Bolognesi, D.P., Matthews, T.J., Emini, E.A., and Putney, S.D., Science 249, 932–935 (1990).

Mandecki, W., Proc. Natl. Acad. Sci. USA 83, 7177–7182 (1986).

Maniatis, T., Fritsch, E.F., and Sambrook, J. In Molecular cloning: a laboratory manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (1982).

Michel, F., Hoffenbach, A., Langlade–Demoyen, P., Guy, B., Lecocq, J.–P., Wain–Hobson, S., Kieny, M.–P., and Plata, F., Eur. J. Immunology 18, 1917–1927 (1988).

Murphy–Corb, M., Martin, L.N., Davison–Fairburn, B., Montelaro, R.C., Miller, M., West, M., Ohkawa, S., Baskin, G.B., Zhang, J.–Y., Putney, S.D., Allison, A.C. and Eppstein, D.A., Science 246, 1293–1297 (1989).

Franchini, G., Fargnoli, K.A., Giomnini, F., Jagodzinski, L., DeRossi, A., Bosch, M., Biberfield, G., Fenyo, E.M., Albert, J., Gallo, R.C., and Wong–Staal, F., Proc. Natl. Acad. Sci. USA 86, 2433–2437 (1989).

Gillard, S., Spehner, D., Drillien, R., and Kirn, A., Proc. Natl. Acad. Sci. USA 83, 5573–5577 (1986).

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., and Paoletti, E., Virology 179, 247–266 (1990a).

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., and Paoletti, E., Virology 179, 517–563 (1990b).

Guo, P., Goebel, S., Davis, S., Perkus, M.E., Taylor, M., Norton, E., Allen, G., Lanquet, B., Desmettre, P., and E Paoletti, J. Virol. 64, 2399–2406 (1990).

Guo, P., Goebel, S., Davis, S., Perkus, M.E., Lanquet, B., Desmettre, P., Allen, G., and Paoletti, E., J. Virol. 63, 4189–4198 (1989).

Haffar, O., Garrigues, J., Travis, B., Moran, P., Zarling, J., and Hu, S.–L., J. Virol. 64, 2653–2659 (1990).

Hu, S.–L., Fultz, P., McClure, H., Eichberg, J., Thomas, E., Zarling, J., Singhal, M., Kosowski, S., Swenson, R., Anderson, D., and Todaro, G., Nature 328, 721–723 (1987).

Hu, S.–L. Travis, B.M., Garrigues, J., Zarling, J.M., Eichberg, J.W. and Alpers, C.E., In Vaccine 90, eds. Chanock, R.M., Lerner, R.A., Brown, F., and Ginsberg, H., (Cold Spring Harbor Press, Cold Spring Harbor, New York) pp. 231–236 (1990).

Hu, S.–L., Kosowski, S., and Dalrymple, J., Nature 320, 537–540, (1986).

Hu, S.L., Zarling, J.M., Chinn, J., Travis, B.M., Moran, P.A., Sias, J., Kuller, L., Morton, W.R., Heidecker, G., and Benveniste, R.E., Proc. Natl. Acad. Sci. USA 86, 7213–7217 (1989).

Javeherian, K., Langlois, A.J., McDanal, C., Ross, K.L., Eckler, L.I., Jellib, C.L., Profy, A.T., Rusche, J.R., Bolognesi, D.P., Putney, S.D., and Matthews, T.J., Proc. Natl. Acad. Sci. USA 86, 6768–6772 (1989).

Behbehani, A.M., Microbiological Reveiws 47, 455–509 (1983).

Bertholet, C., Drillien, R. and Wittek, R., Proc. Natl. Acad. Sci. 82, 2096–2100 (1985).

Chakrabarti, S., Robert–Guroff, M., Wong–Staal, F., Gallo, R.C., and Moss, B., Nature 320, 535–537 (1986).

Cianciolo, G.J., Copeland T.D., Oroszlan S., and Snyderman, R., Science 230, 453–456 (1985).

Clewell, D.B., and Helinski, D.R., Proc. Natl. Acad. Sci. USA 62, 1159–1166 (1969).

Clewell, D.B., J. Bacteriol 110, 667–676 (1972).

Colinas, R.J., Condit, R.C., and Paoletti, E., Virus Research 18, 49–70 (1990).

Desrosiers, R.C., M.S., Wyand, T., Kodama, T.J. Ringler, L.O. Arthur, P.K. Seghal, N.L. Letvin, N.W. King, and M.D. Daniel, Proc. Natl. Acad. Sci. USA 86, 6353–6357 (1989).

Dreyfuss, G., Adam, S.A., and Choi, Y.–D., Mol. Cell. Biol. 4, 415–423 (1984).

Engelke, D.R., Hoener, P.A., and Collins, F.S., Proc. Natl. Acad. Sci. USA 85, 544–548 (1988).

Franchini, G., Gurgo, C., Guo, H.–G., Gallo, R.C., Collati, E., Fargnoli, K.A., Hall, L.F., Wong–Staal, F., and Reitz, Jr., M.S., Nature (London) 328, 539–543 (1987).

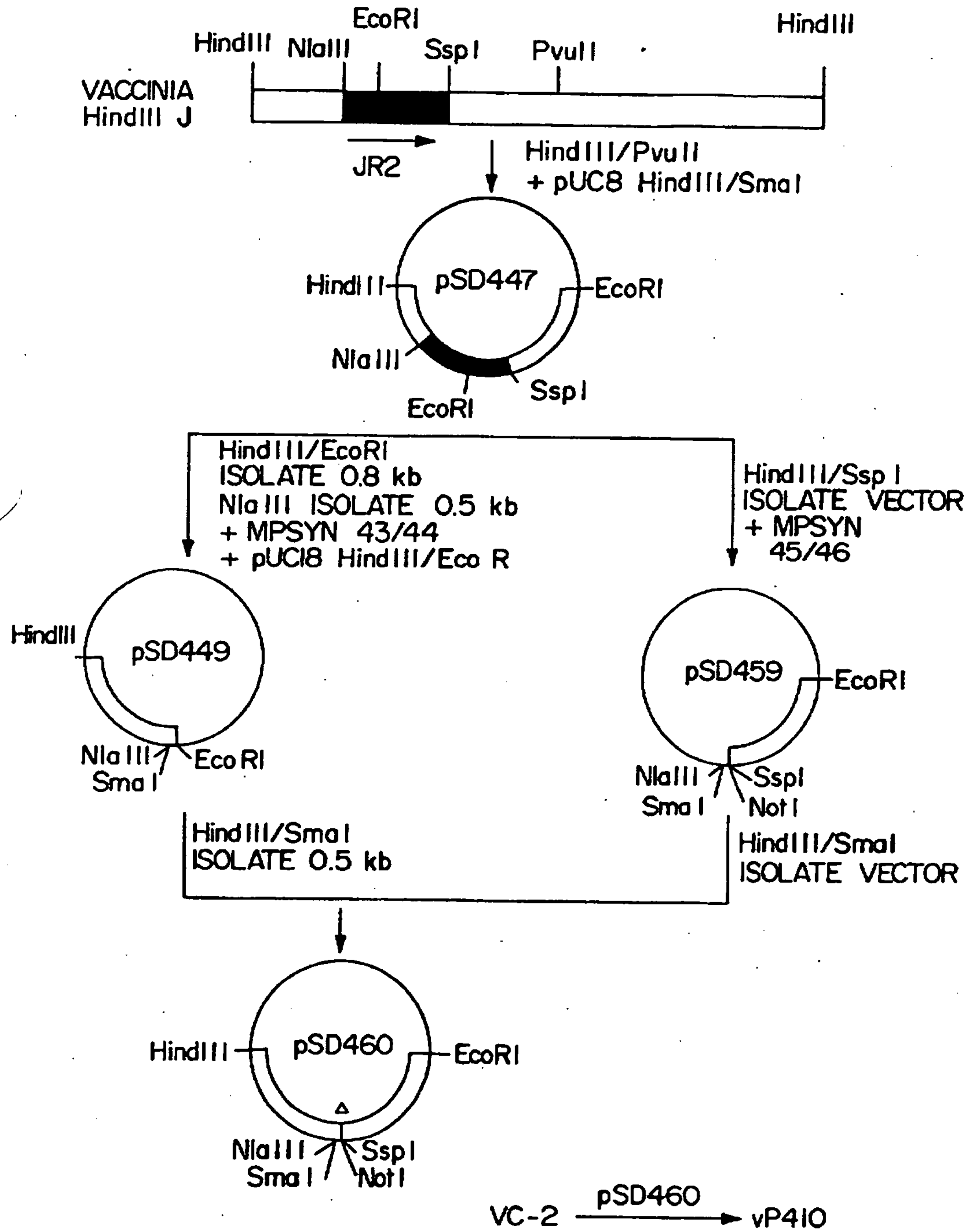

VACCINIA
HindIII J
HindIII
NlaIII
EcoRI
SspI
PvuII
HindIII
JR2
HindIII/PvuII
+ pUC8 HindIII/SmaI
pSD447
HindIII
EcoRI
NlaIII
EcoRI
SspI
HindIII/EcoRI
ISOLATE 0.8 kb
NlaIII ISOLATE 0.5 kb
+ MPSYN 43/44
+ pUC18 HindIII/Eco R
HindIII/Ssp I
ISOLATE VECTOR
+ MPSYN
45/46
pSD449
HindIII
NlaIII
SmaI
EcoRI
pSD459
EcoRI
NlaIII
SmaI
SspI
NotI
HindIII/SmaI
ISOLATE 0.5 kb
HindIII/SmaI
ISOLATE VECTOR
pSD460
HindIII
EcoRI
NlaIII
SmaI
SspI
NotI
VC-2
pSD460
vP410

FIG. 1

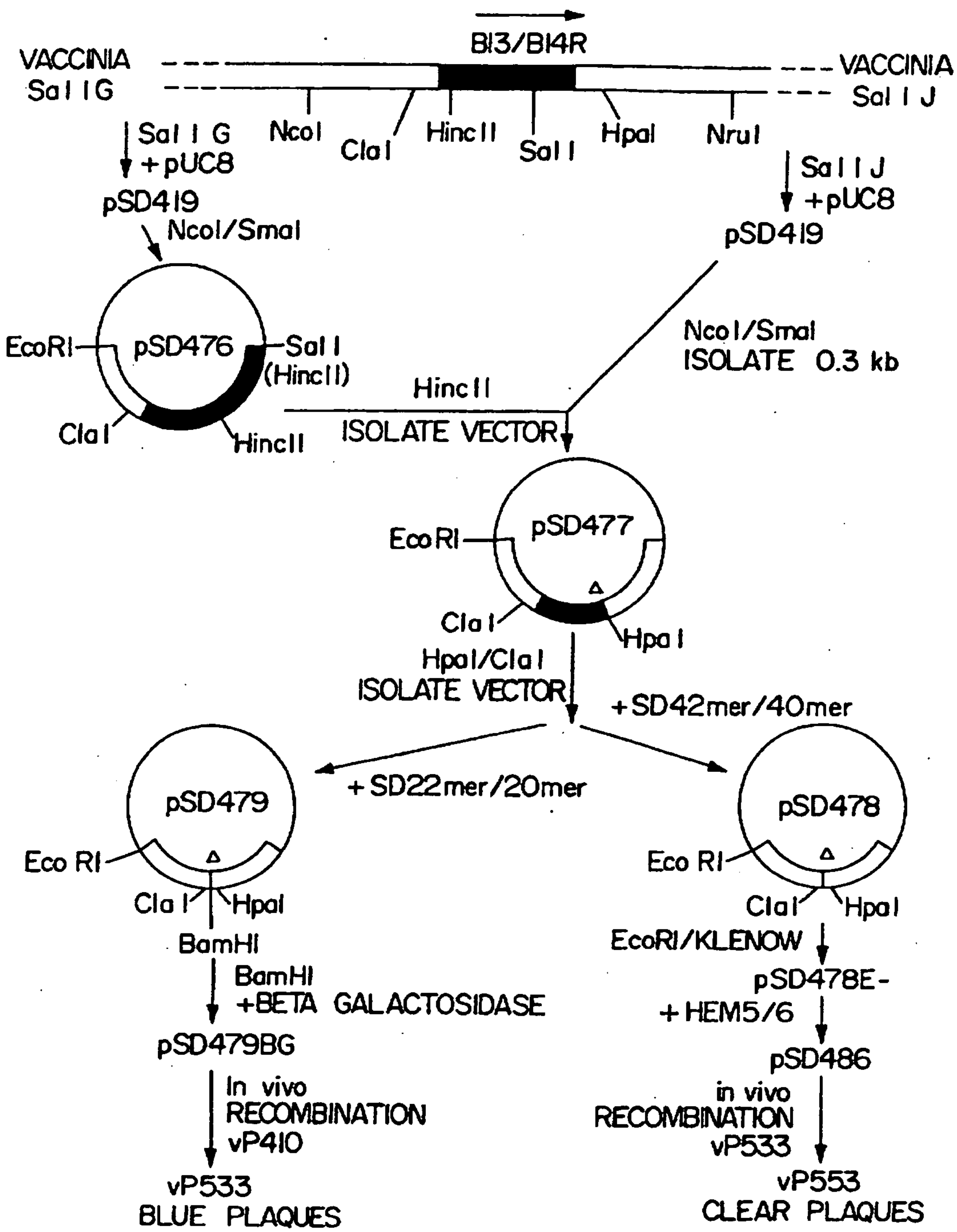

B13/B14R
VACCINIA
Sal I G
VACCINIA
Sal I J
NcoI
ClaI
HincII
Sal I
HpaI
NruI
Sal I G
+pUC8
pSD419
NcoI/SmaI
Sal I J
+pUC8
pSD419
EcoRI
pSD476
Sal I
(HincII)
ClaI
HincII
NcoI/SmaI
ISOLATE 0.3 kb
HincII
ISOLATE VECTOR
EcoRI
pSD477
ClaI
HpaI
HpaI/ClaI
ISOLATE VECTOR
+SD42mer/40mer
+SD22mer/20mer
pSD479
Eco RI
ClaI
HpaI
BamHI
BamHI
+BETA GALACTOSIDASE
pSD479BG
In vivo
RECOMBINATION
vP410
vP533
BLUE PLAQUES
pSD478
Eco RI
ClaI
HpaI
EcoRI/KLENOW
pSD478E-
+HEM5/6
pSD486
in vivo
RECOMBINATION
vP533
vP553
CLEAR PLAQUES

FIG. 2

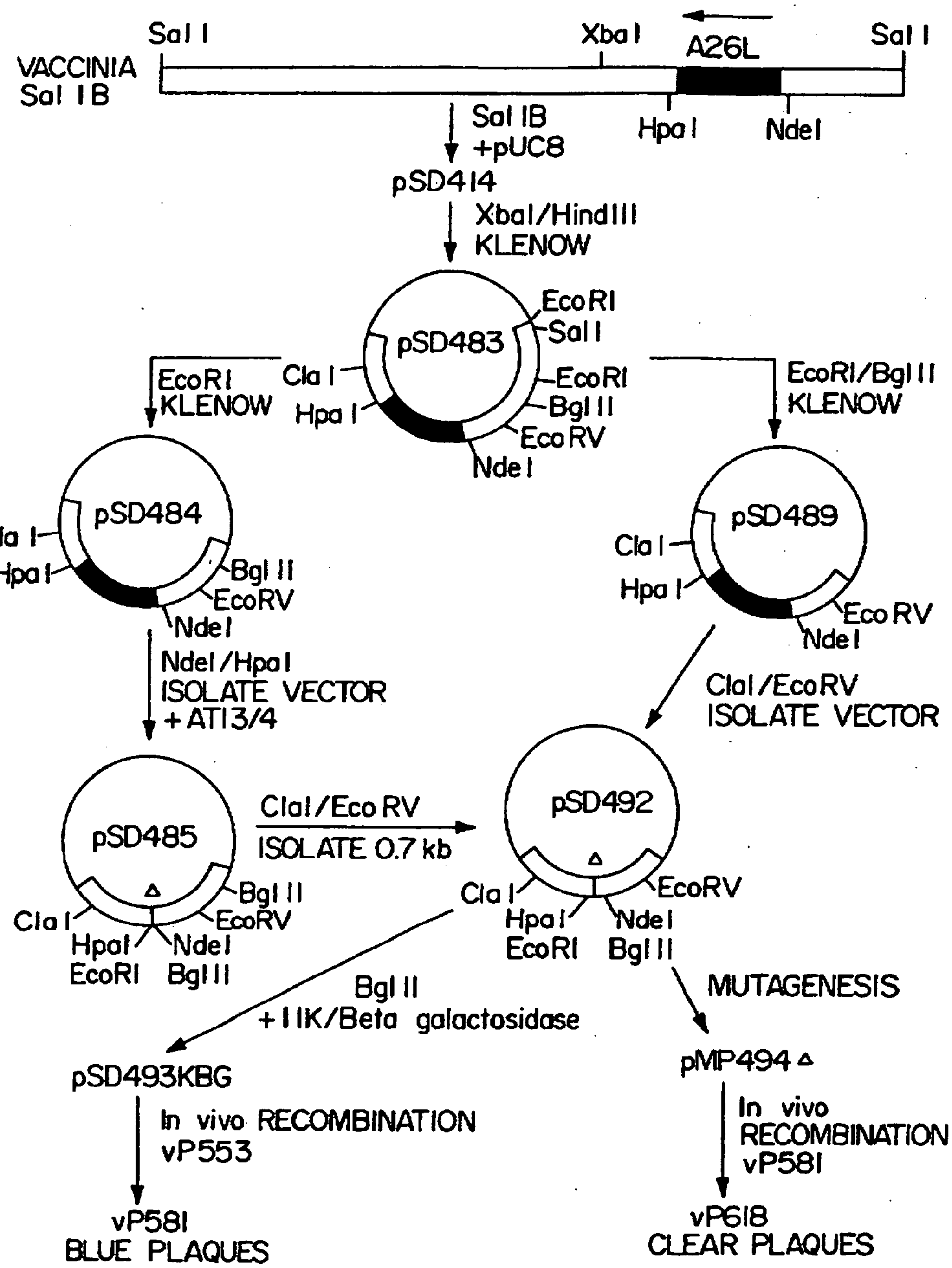

Sal I
XbaI
A26L
Sal I
VACCINIA
Sal IB
Hpa I
NdeI
Sal IB
+pUC8
pSD414
XbaI/HindIII
KLENOW
pSD483
EcoRI
Sal I
Cla I
EcoRI
Bgl II
Hpa I
EcoRV
NdeI
EcoRI
KLENOW
EcoRI/BglII
KLENOW
pSD484
Cla I
Hpa I
Bgl II
EcoRV
NdeI
pSD489
Cla I
Hpa I
Eco RV
NdeI
NdeI/HpaI
ISOLATE VECTOR
+ ATI3/4
ClaI/EcoRV
ISOLATE VECTOR
pSD485
Bgl II
Cla I
EcoRV
HpaI
NdeI
EcoRI
BglII
ClaI/Eco RV
ISOLATE 0.7 kb
pSD492
Cla I
EcoRV
Hpa I
NdeI
EcoRI
BglII
Bgl II
+ 11K/Beta galactosidase
MUTAGENESIS
pSD493KBG
pMP494
In vivo RECOMBINATION
vP553
In vivo
RECOMBINATION
vP581
vP581
BLUE PLAQUES
vP618
CLEAR PLAQUES

FIG. 3

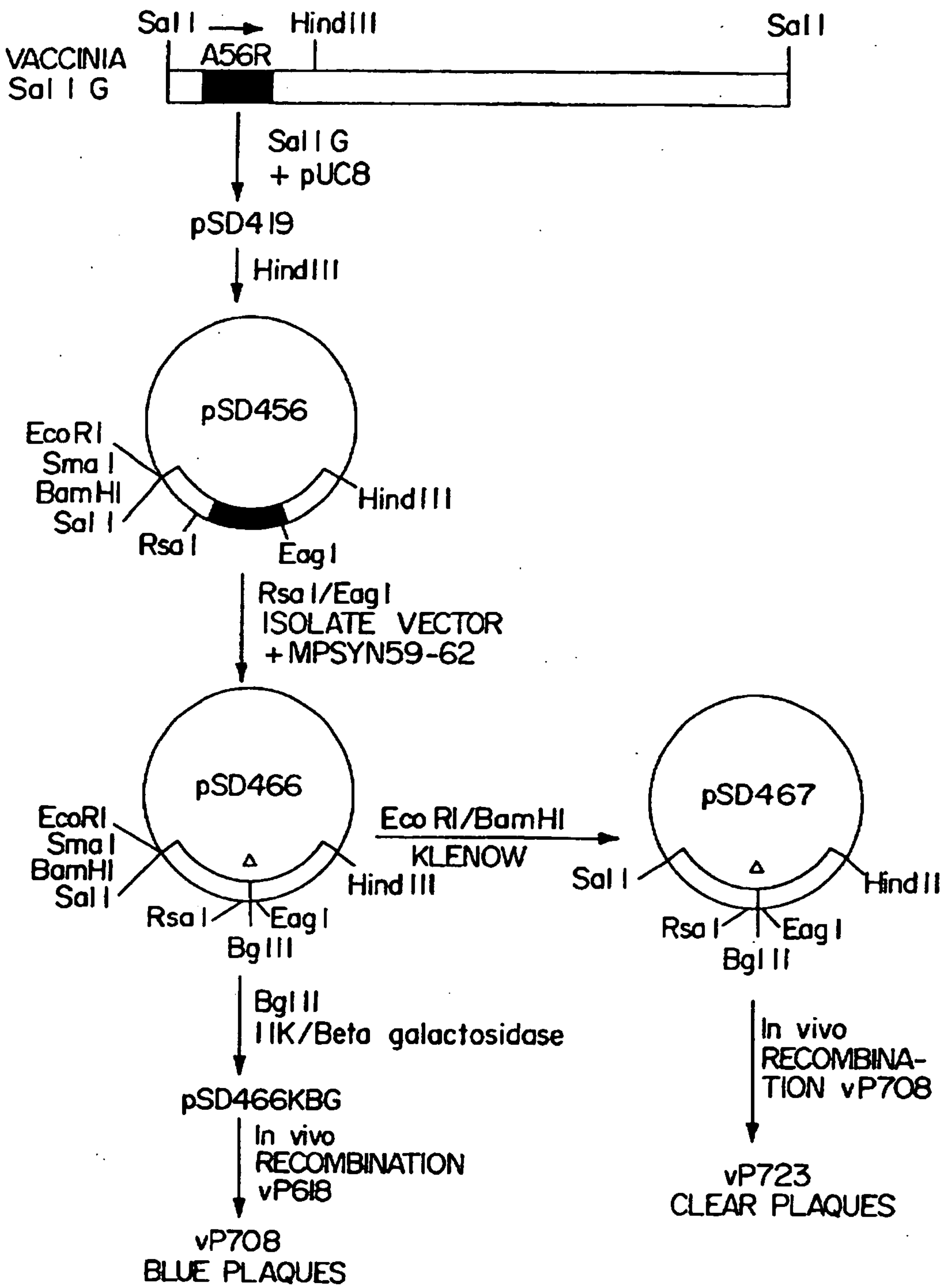

Sal I
Hind III
Sal I
VACCINIA
Sal I G
A56R
Sal I G
+ pUC8
pSD419
Hind III
pSD456
EcoRI
Sma I
BamHI
Sal I
Rsa I
Eag I
Hind III
Rsa I/Eag I
ISOLATE VECTOR
+MPSYN59-62
pSD466
EcoRI
Sma I
BamHI
Sal I
Rsa I
Eag I
Bg III
Hind III
Eco RI/BamHI
KLENOW
pSD467
Sal I
Hind III
Rsa I
Eag I
Bgl II
Bgl II
11K/Beta galactosidase
pSD466KBG
In vivo
RECOMBINATION
vP618
vP708
BLUE PLAQUES
In vivo
RECOMBINA-
TION vP708
vP723
CLEAR PLAQUES

FIG. 4

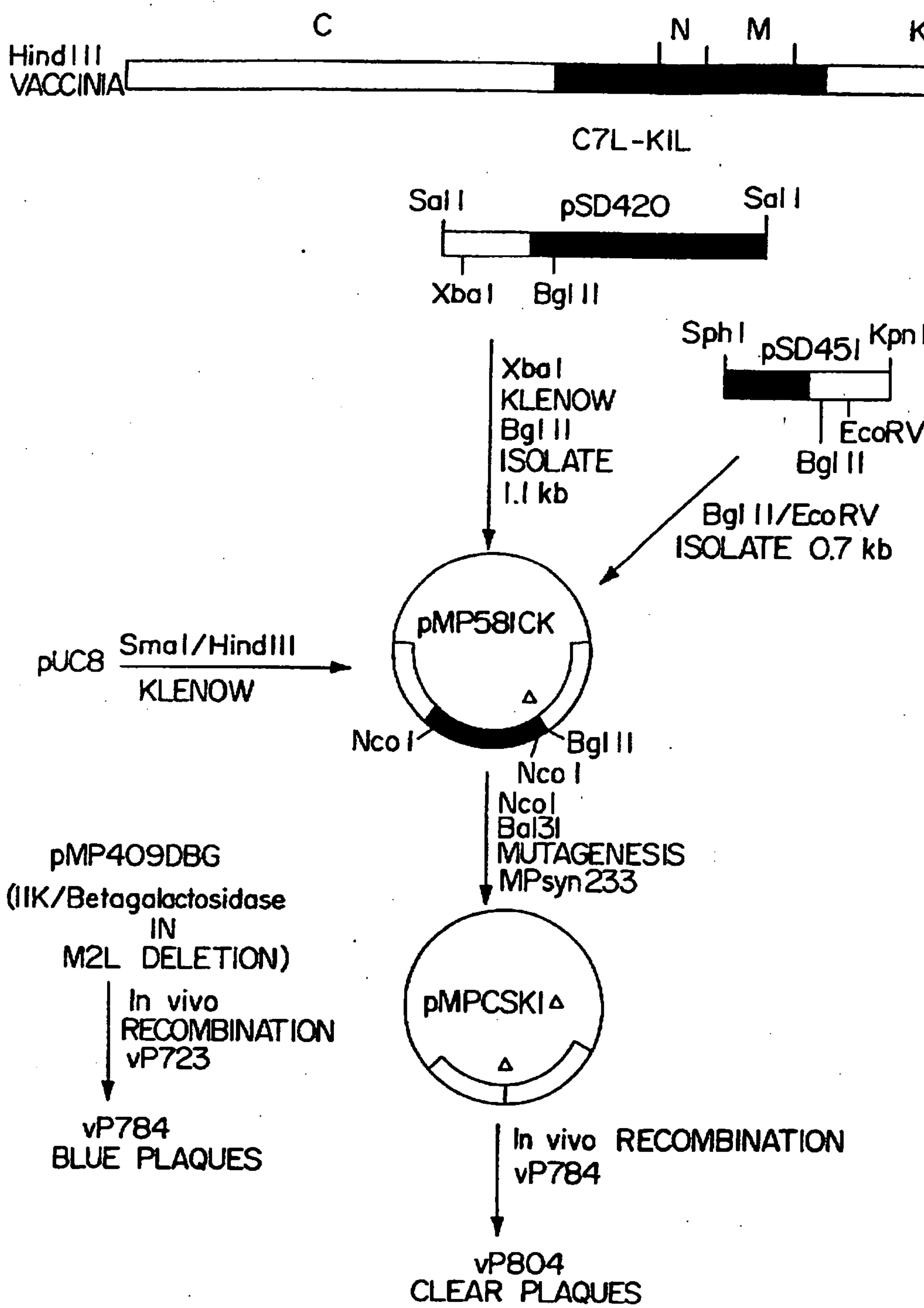
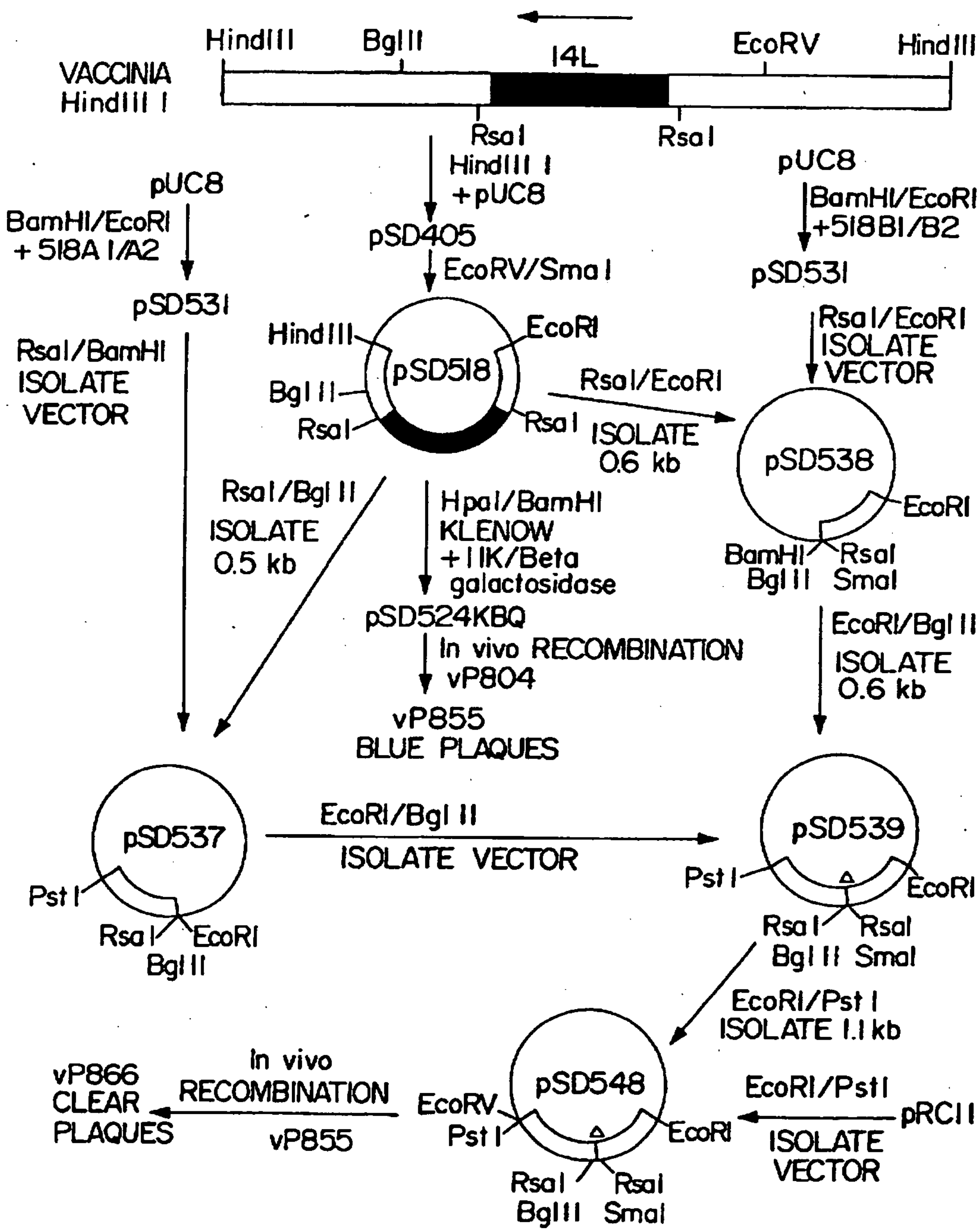

HindIII
VACCINIA
C
N
M
K
C7L-KIL
SalI
pSD420
SalI
XbaI
BglII
SphI
pSD451
KpnI
EcoRV
BglII
XbaI
KLENOW
BglII
ISOLATE
1.1 kb
BglII/EcoRV
ISOLATE 0.7 kb
pUC8
SmaI/HindIII
KLENOW
pMP581CK
NcoI
BglII
NcoI
NcoI
Bal31
MUTAGENESIS
MPsyn233
pMP409DBG
(IIK/Betagalactosidase
IN
M2L DELETION)
In vivo
RECOMBINATION
vP723
vP784
BLUE PLAQUES
pMPCSK1Δ
In vivo RECOMBINATION
vP784
vP804
CLEAR PLAQUES

FIG. 5



HindIII
BglII
14L
EcoRV
HindIII
VACCINIA
HindIII I
RsaI
RsaI
HindIII I
+pUC8
pSD405
EcoRV/SmaI
pUC8
BamHI/EcoRI
+518A1/A2
pSD531
RsaI/BamHI
ISOLATE
VECTOR
pUC8
BamHI/EcoRI
+518B1/B2
pSD531
RsaI/EcoRI
ISOLATE
VECTOR
HindIII
EcoRI
pSD518
BglII
RsaI
RsaI
RsaI/EcoRI
ISOLATE
0.6 kb
pSD538
EcoRI
BamHI
BglII
RsaI
SmaI
RsaI/BglII
ISOLATE
0.5 kb
HpaI/BamHI
KLENOW
+11K/Beta
galactosidase
pSD524KBQ
In vivo RECOMBINATION
vP804
vP855
BLUE PLAQUES
EcoRI/BglII
ISOLATE
0.6 kb
pSD537
PstI
RsaI
EcoRI
BglII
EcoRI/BglII
ISOLATE VECTOR
pSD539
PstI
EcoRI
RsaI
RsaI
BglII
SmaI
EcoRI/PstI
ISOLATE 1.1 kb
vP866
CLEAR
PLAQUES
In vivo
RECOMBINATION
vP855
EcoRV
PstI
pSD548
EcoRI
RsaI
BglII
RsaI
SmaI
EcoRI/PstI
pRC11
ISOLATE
VECTOR

FIG. 6

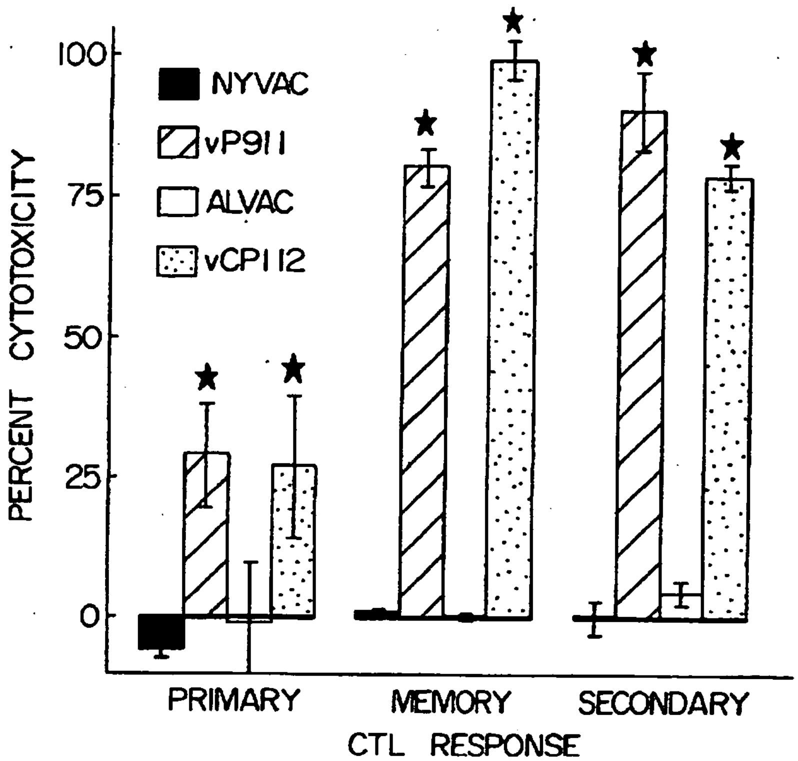

100
75
50
25
0
PERCENT CYTOTOXICITY
NYVAC
vP911
ALVAC
vCP112
PRIMARY
MEMORY
SECONDARY
CTL RESPONSE

FIG. 7

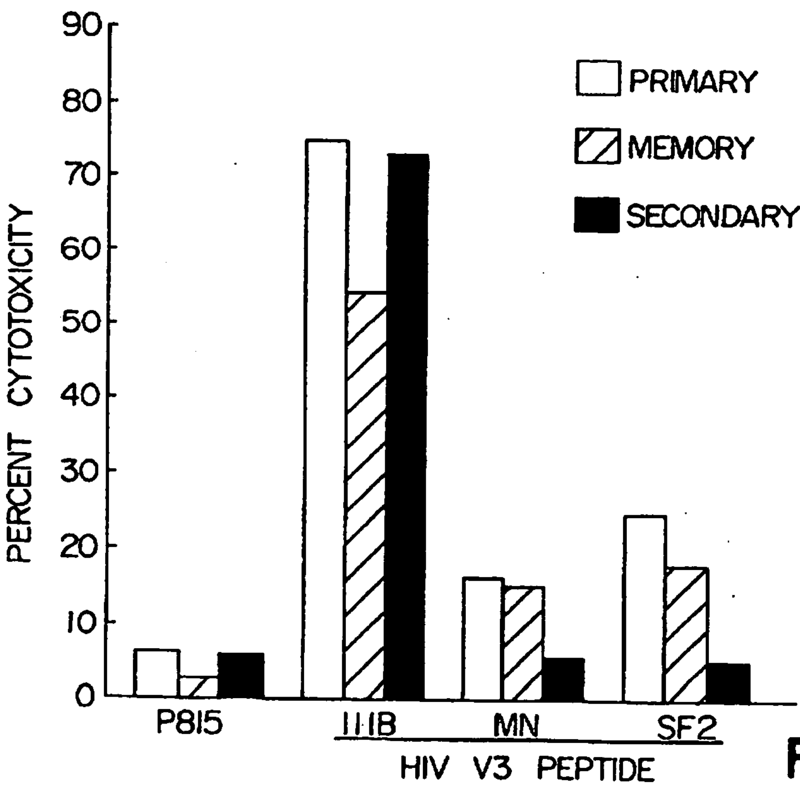

90
80
70
60
50
40
30
20
10
0
PERCENT CYTOTOXICITY
PRIMARY
MEMORY
SECONDARY
P815
IIIB
MN
SF2
HIV V3 PEPTIDE

FIG. 8

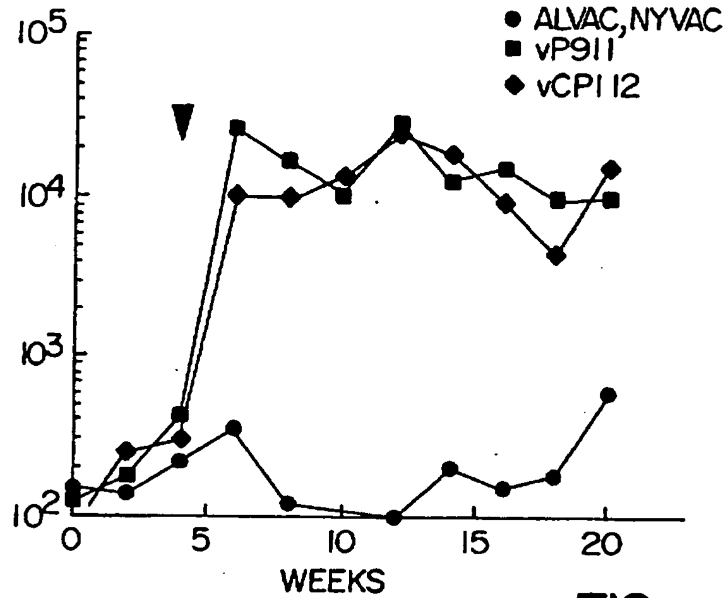

ALVAC, NYVAC
vP911
vCP112
10^5
10^4
10^3
10^2
0
5
10
15
20
WEEKS

FIG. 9

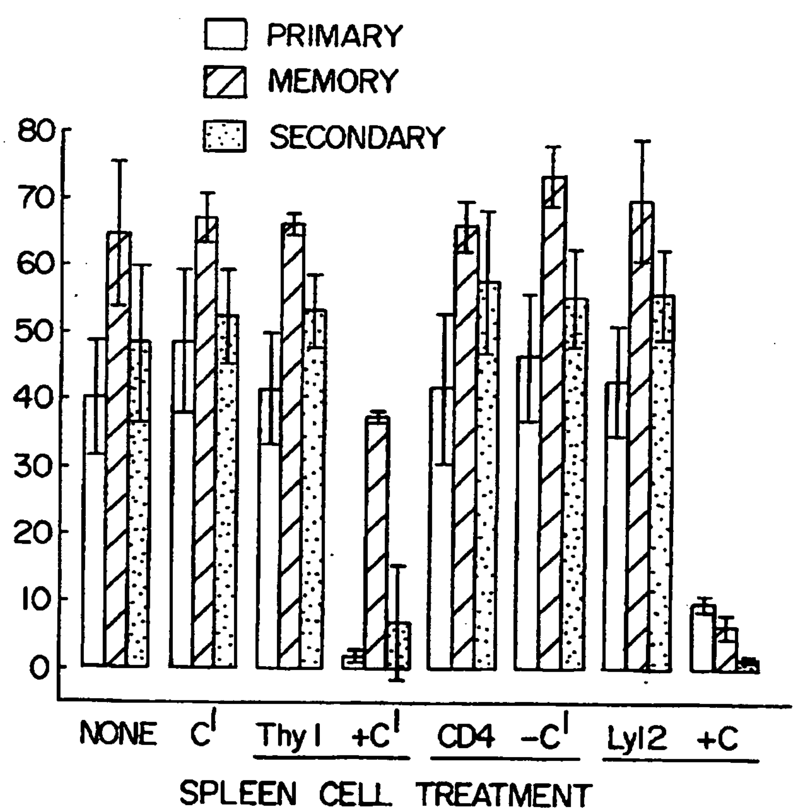

PRIMARY
MEMORY
SECONDARY
80
70
60
50
40
30
20
10
0
NONE
C'
Thy1 +C'
CD4 -C'
Ly12 +C
SPLEEN CELL TREATMENT

FIG. 10

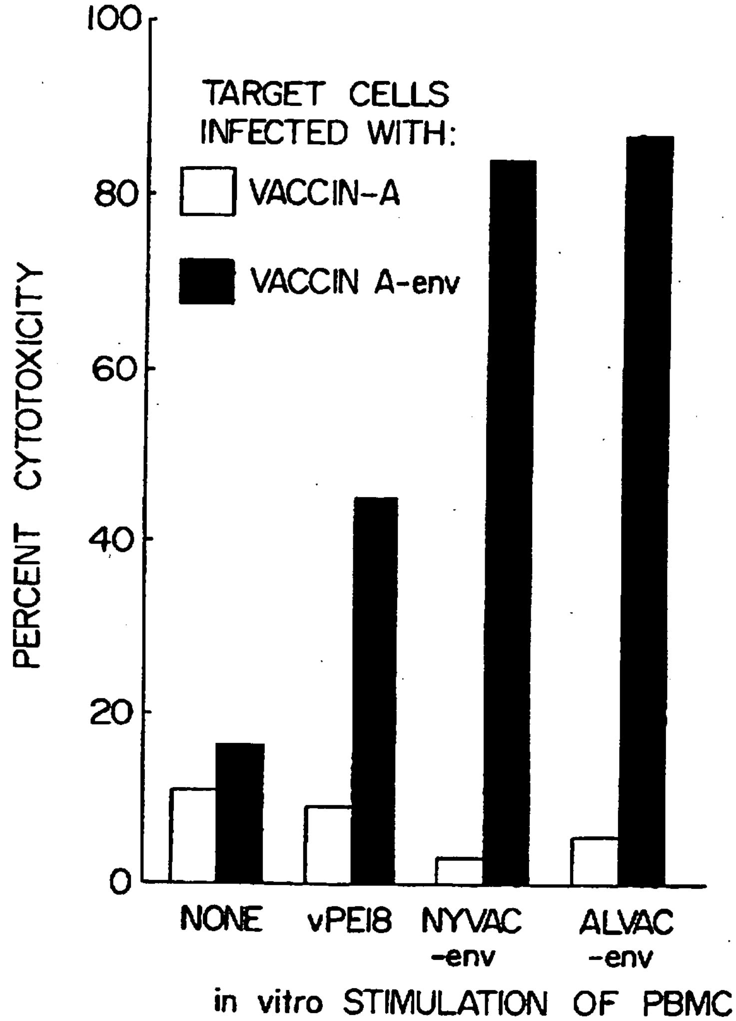

100
80
60
40
20
0
PERCENT CYTOTOXICITY
TARGET CELLS INFECTED WITH:
VACCIN-A
VACCIN A-env
NONE
vPE18
NYVAC -env
ALVAC -env
in vitro STIMULATION OF PBMC

FIG. 11

RECOMBINANT ATTENUATED ALVAC CANARYPOXVIRUS EXPRESSION VECTORS CONTAINING HETEROLOGOUS DNA SEGMENTS ENCODING LENTIVIRAL GENE PRODUCTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of application Ser. No. 07/897,382, filed Jun. 11, 1992 now abandoned, which in turn is a continuation-in-part of application Ser. No. 07/715,921, filed Jun. 14, 1991, now abandoned. Application Ser. No. 07/897,382 is also a continuation-in-part of copending application Ser. No. 07/847,951, filed Mar. 6, 1992, also incorporated herein by reference application Ser. No. 07/847,951 is a continuation-in-part of application Ser. No. 07/713,967, filed Jun. 11, 1991, which in turn is a continuation-in-part of application Ser. No. 07/666,056, filed Mar. 7, 1991.

FIELD OF THE INVENTION

The present invention relates to a modified poxvirus and to methods of making and using the same. More in particular, the invention relates to recombinant poxvirus, which virus expresses gene products of an immunodeficiency virus gene, and to immunogenic compositions which induce an immunological response against immunodeficiency virus infections when administered to a host.

Several publications are referenced in this application. Full citation to these references is found at the end of the specification preceding the claims. These references describe the state-of-the-art to which this invention pertains.

BACKGROUND OF THE INVENTION

Vaccinia virus and more recently other poxviruses have been used for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into live infectious poxvirus involves recombination between pox DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus (Piccini et al., 1987).

Specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of the vaccinia virus described in U.S. Pat. Nos. 5,110,587, 4,769,330, 4,722,848 and 4,603,112, the disclosures of which are incorporated herein by reference. In this regard, reference is also made to copending U.S. applications Ser. Nos. 881, 995 filed May 4, 1992 and 537,890, filed Jun. 14, 1990, also incorporated herein by reference.

First, the DNA gene sequence to be inserted into the virus, particularly an open reading frame from a non-pox source, is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1986).

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, in a nonessential region of its genome, of foreign DNA sequences. The term "foreign" DNA designates exogenous DNA, particularly DNA from a non-pox source, that codes for gene products not ordinarily produced by the genome into which the exogenous DNA is placed.

Genetic recombination is in general the exchange of homologous sections of DNA between two strands of DNA. In certain viruses RNA may replace DNA. Homologous sections of nucleic acid are sections of nucleic acid (DNA or RNA) which have the same sequence of nucleotide bases.

Genetic recombination may take place naturally during the replication or manufacture of new viral genomes within the infected host cell. Thus, genetic recombination between viral genes may occur during the viral replication cycle that takes place in a host cell which is co-infected with two or more different viruses or other genetic constructs. A section of DNA from a first genome is used interchangeably in constructing the section of the genome of a second co-infecting virus in which the DNA is homologous with that of the first viral genome.

However, recombination can also take place between sections of DNA in different genomes that are not perfectly homologous. If one such section is from a first genome homologous with a section of another genome except for the presence within the first section of, for example, a genetic marker or a gene coding for an antigenic determinant inserted into a portion of the homologous DNA, recombination can still take place and the products of that recombination are then detectable by the presence of that genetic marker or gene in the recombinant viral genome.

Successful expression of the inserted DNA genetic sequence by the modified infectious virus requires two conditions. First, the insertion must be into a nonessential region of the virus in order that the modified virus remain viable. The second condition for expression of inserted DNA is the presence of a promoter in the proper relationship to the inserted DNA. The promoter must be placed so that it is located upstream from the DNA sequence to be expressed.

In recent years much attention within the field of medical virology has been focused on the escalating incidence of acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV). HIV-1 is a member of the virus family, Retroviridae, and more specifically of the Lentivirus subfamily. This viral system along with other related viruses such as HIV-2 and simian immunodeficiency virus (SIV) have been scrutinized with respect to their molecular biology, immunology, and pathogenesis in an effort to develop safe and efficacious vaccines and antiviral therapies. Two major obstacles, however, have haunted the design of HIV vaccine candidates. For one, there exists considerable sequence heterogeneity among HIV isolates, and secondly, there exists a lack of information pertaining to protective immunity. Infected individuals develop antibody (Starcich et al., 1986; Weiss et al., 1985) and $CD8^+$-T cell (Walker et al., 1987; Plata et al., 1987) responses to HIV but are not protected since they eventually develop AIDS.

Vaccinia virus has been used extensively to express a number of HIV gene products to investigate their biochemical, functional and immunological properties, particularly cell-mediated responses. Using these specific reagents, cell-mediated cytotoxic activities have been demonstrated in seropositive individuals towards the envelope (Riviere et al., 1989; Walker et al., 1987; Koup et al., 1989), core proteins (gag) (Riviere et al., 1989; Koup et al., 1989,

Nixon et al., 1988) pol (Walker et al., 1989; Walker et al., 1988), and the nef (Riviere et al., 1989) gene products. Additionally, vaccinia virus recombinants containing HIV genes have been shown to elicit both cell-mediated and humoral immune responses in small laboratory animals (Chakrabarti et al., 1986; Hu et al., 1986; Rautman et al., 1989; Michel et al., 1988), macaques (Zarling et al., 1986), chimpanzees (Hu et al., 1987) and, significantly, humans (Zagury et al., 1988; Koff et al., 1989). To date, limited vaccination/protection studies in primates have been reported with vaccinia virus recombinants expressing gene products from HIV and related viruses.

Vaccination of primates with a recombinant vaccinia virus expressing the envelope glycoprotein from AIDS-causing retroviruses have elicited humoral and cell-mediated immune responses and, more significantly, have protected against HIV infection (Zagury et al., 1988; Hu et al., 1989). In the results described by Hu et al., a vaccinia virus recombinant expressing the envelope glycoprotein from simian AIDS retrovirus (SRV-2) was used to vaccinate pig-tailed macaques (*Macaca nemestrina*). All immunized animals developed both SRV-specific cell-mediated and humoral immune responses, including neutralizing antibodies and antibodies which mediate ADCC towards SRV-2 infected cells. Animals were challenged intravenously with 5×10^3 $TCID_{50}$ of SRV-2/W. The challenged control animals (non-vaccinated) became viremic by two weeks post-challenge and those that did not seroconvert died by seven weeks post-challenge. Significantly, all of the challenged animals which were previously inoculated with the vaccinia virus-env recombinant remained healthy, virus-free and seropositive exclusively against the envelope antigen.

In a pilot experiment in humans, HIV seronegative individuals were vaccinated with a vaccinia virus/HIV-1 envelope recombinant. This primary inoculation resulted in weak immunological responses (Zagury et al., 1988). These primary responses were subsequently boosted with various protocols. The use of an intravenous injection of paraformaldehyde-fixed autologous cells infected in vitro with the vaccinia virus-HIV recombinant, however, provided the most significant booster effect. With this immunization protocol, anamnestic immune responses were achieved against the envelope antigen consisting of group-specific neutralizing antibodies, cytotoxic T-lymphocytes and delayed-type hypersensitivity (Zagury et al., 1988). In clinical trials performed in the United States using a recombinant vaccinia virus expressing the HIV env gene (HIVAVle; Bristol-Meyers), individuals receiving the recombinant mounted T-cell proliferative responses to HIV (Koff et al., 1989). The intensity of these responses, however, was affected by prior exposure to vaccinia virus. Consequently, individuals immune to vaccinia virus mounted a weak and transient T-cell response whereas in individuals not immune to vaccinia virus, a strong response was observed towards the HIV envelope antigen (reviewed by Koff et al., 1989). These results are encouraging and have provided evidence that an immune state can be obtained in man prior to HIV exposure using a poxvirus-based immunization vehicle.

An intriguing potential in terms of HIV-1 vaccinology is provided by expression of the HIV-1 gag products by vaccinia virus either alone or in combination with the envelope glycoprotein. Several laboratories have demonstrated that expression of gag or gag/pol sequences by vaccinia virus results in the production of non-infectious particles (Hu et al., 1990; Shioda et al., 1990; Karacostas et al., 1989). Analysis of these vaccinia virus recombinant infected cells by electron microscopy revealed retrovirus-like particles budding from the plasma membrane and extracellular forms which were indistinguishable from particles observed in HIV-1 infected cells. Biochemically, these particles are also similar to native HIV-1 particles. Moreover, rodents and chimpanzees inoculated with a vaccinia virus-HIV gag recombinant generated both humoral and cell-mediated immune responses to the HIV-1 core antigens (Hu et al., 1990).

The co-expression of the HIV-1 envelope glycoprotein with the core proteins in mammalian cells by vaccinia virus also resulted in the assembly and release of HIV-1 particles (Haffar et al., 1990). These typical type C-retrovirus particles contained both the envelope glycoproteins (gp120 and gp41) and the gag proteins (p24, p17, p55, and p39). These proteins were also described as being present in these recombinant-made particles in the same ratio as observed in HIV-1 virions. The production of these non-infectious HIV particles either in purified form or synthesized in a vaccinee upon inoculation with a multivalent vaccinia virus recombinant may provide valuable "whole-virus" vaccinating agents against HIV. This is especially appealing since protection against the lentivirus, SIV, has been demonstrated using whole inactivated virus preparations (Murphy-Corb et al., 1989; Derosiers et al., 1989).

The above reviewed examples demonstrate the potential use of vaccinia virus recombinants expressing HIV antigens to induce pertinent immunological responses necessary for protection. A major concern, however, about the use of live viral vaccines is the issue of safety. Rare complications from immunization with vaccinia virus have been documented, particularly in immunocompromised individuals, and have raised some objections for their acceptance as vaccine candidates (Behbehani, 1983; Lane et al., 1969). Recently, however, significant strides have been made in understanding viral virulence factors with the hope of modifying strains for use as immunization vehicles (Tartaglia et al., 1990). With more relevance to the development of vaccinia virus-based HIV vaccine candidates for the immunoprophylaxis and immunotherapy of AIDS, the genes responsible for the productive replication of vaccinia virus in human cell systems have been identified (Gillard et al., 1986; Perkus et al., 1990). Potential use of vaccinia virus vectors devoid of these genes provide a non-replicating vector vaccine candidate that may present appropriate HIV antigens in a fashion that elicits a protective immune response.

Another approach towards the generation of safe and effective poxvirus based HIV vaccine candidates utilize avipoxvirus vectors (i.e. canarypoxvirus and fowlpoxvirus) to express pertinent HIV antigens. These viruses are naturally host-restricted and only productively replicate in avian species. Therefore, there exists a built-in safety factor for their use in humans, particularly immunocompromised individuals. In this regard, these viruses have been engineered to express the rabies G glycoprotein and have been demonstrated to protect various nonavian species from live rabies challenge (Taylor et al., 1988a; 1991).

It can thus be appreciated that provision of an immunodeficiency virus recombinant poxvirus, and of an immunogenic composition which induces an immunological response against immunodeficiency virus infections when administered to a host, particularly a composition having enhanced safety, would be a highly desirable advance over the current state of technology.

OBJECTS OF THE INVENTION

It is therefore an object of this invention to provide recombinant poxviruses, which viruses express gene prod-

ucts of an immunodeficiency virus, and to provide a method of making such recombinant poxviruses.

It is an additional object of this invention to provide for the cloning and expression of immunodeficiency virus coding sequences, particularly human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) coding sequences in a poxvirus vector.

It is another object of this invention to provide an immunological composition having enhanced safety and which is capable of inducing an immunological response against immunodeficiency virus infections when administered to a host.

These and other objects and advantages of the present invention will become more readily apparent after consideration of the following.

STATEMENT OF THE INVENTION

In one aspect, the present invention relates to a recombinant poxvirus containing therein a DNA sequence from an immunodeficiency virus, particularly HIV or SIV, in a nonessential region of the poxvirus genome. The poxvirus is advantageously a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus.

According to the present invention, the recombinant poxvirus expresses gene products of the foreign immunodeficiency virus gene, particularly an HIV or SIV gene.

In another aspect, the present invention relates to a vaccine for inducing an immunological response in a host animal inoculated with the vaccine, said vaccine including a carrier and a recombinant poxvirus containing, in a nonessential region thereof, DNA from an immunodeficiency virus, particularly HIV or SIV. The poxvirus used in the vaccine according to the present invention is advantageously a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus.

BRIEF DESCRIPTION OF THE DRAWINGS

A better understanding of the present invention will be had by referring to the accompanying drawings, in which:

FIG. 1 schematically shows a method for the construction of plasmid pSD460 for deletion of thymidine kinase gene and generation of recombinant vaccinia virus vP410;

FIG. 2 schematically shows a method for the construction of plasmid pSD486 for deletion of hemorrhagic region and generation of recombinant vaccinia virus vP553;

FIG. 3 schematically shows a method for the construction of plasmid pMP494Δ for deletion of ATI region and generation of recombinant vaccinia virus vP618;

FIG. 4 schematically shows a method for the construction of plasmid pSD467 for deletion of hemagglutinin gene and generation of recombinant vaccinia virus vP723;

FIG. 5 schematically shows a method for the construction of plasmid pMPCSK1Δ for deletion of gene cluster [C7L–K1L] and generation of recombinant vaccinia virus vP804;

FIG. 6 schematically shows a method for the construction of plasmid pSD548 for deletion of large subunit, ribonucleotide reductase and generation of recombinant vaccinia virus vP866 (NYVAC);

FIG. 7 shows the cytotoxic response of spleen cells of mice and immunized with vaccinia virus or canarypox virus vectors (NYVAC, ALVAC) or with vaccinia virus or canarypox virus recombinants expressing HIV III B env (vP911, vCP112);

FIG. 8 shows the specificity of cytotoxic T lymphocyte antigen receptor for the HIV II B hypervariable V3 loop of gp120, but not for the V3 loop of HIV MN or SF2;

FIG. 9 shows the antibody responses to HIV III B gp120 of mice immunized with vectors (NYVAC, ALVAC) or with vaccinia virus recombinant vP911 or canarypox recombinant vCP112 expressing HIV-1 env (inverted triangle indicates time of administration of second inoculation);

FIG. 10 shows the sensitivity of the cytotoxic effector cells from the spleens of mice immunized with vCP112 to antibodies against cytotoxic T lymphocyte cell surface antigens Thy 1.2 and Lyt 2.2; and

FIG. 11 shows percent cytotoxity versus in vitro stimulation of PBMC.

DETAILED DESCRIPTION OF THE INVENTION

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

In the Examples, the following methods and materials are employed.

DNA Cloning and Synthesis

Plasmids were constructed, screened and grown by standard procedures (Maniatis et al., 1982; Perkus et al., 1985; Piccini et al., 1987). Restriction endonucleases were obtained from GIBCO/BRL, Gaithersburg, Md.; New England Biolabs, Beverly, Mass.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind. Klenow fragment of *E. coli* polymerase was obtained from Boehringer Mannheim Biochemicals. BAL-31 exonuclease and phage T4 DNA ligase were obtained from New England Biolabs. The reagents were used as specified by the various suppliers.

Synthetic oligodeoxyribonucleotides were prepared on a Biosearch 8750 or Applied Biosystems 380B DNA synthesizer as previously described (Perkus et al., 1989). DNA sequencing was performed by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase (Tabor et al., 1987) as previously described (Guo et al., 1989). DNA amplification by polymerase chain reaction (PCR) for sequence verification (Engelke et al., 1988) was performed using custom synthesized oligonucleotide primers and GeneAmp DNA amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, Conn.) in an automated Perkin Elmer Cetus DNA Thermal Cycler. Excess DNA sequences were deleted from plasmids by restriction endonuclease digestion followed by limited digestion by BAL-31 exonuclease and mutagenesis (Mandecki, 1986) using synthetic oligonucleotides.

Cells, Virus, and Transfection

The origins and conditions of cultivation of the Copenhagen strain of vaccinia virus has been previously described (Guo et al., 1989). Generation of recombinant virus by recombination, in situ hybridization of nitrocellulose filters and screening for B-galactosidase activity are as previously described (Panicali et al., 1982; Perkus et al., 1989).

The parental canarypox virus (Rentschler strain) is a vaccinal strain for canaries. The vaccine strain was obtained from a wild type isolate and attenuated through more than 200 serial passages on chick embryo fibroblasts. A master viral seed was subjected to four successive plaque purifications under agar and one plaque clone was amplified through five additional passages after which the stock virus was used as the parental virus in in vitro recombination tests. The plaque purified canarypox isolate is designated ALVAC. ALVAC was deposited under the terms of the Budapest Treaty with the ATCC, accession number VR-2547.

The strain of fowlpox virus (FPV) designated FP-1 has been described previously (Taylor et al., 1988a). It is an attenuated vaccine strain useful in vaccination of day old chickens. The parental virus strain Duvette was obtained in France as a fowlpox scale from a chicken. The virus was attenuated by approximately 50 serial passages in chicken embryonated eggs followed by 25 passages on chicken embryo fibroblast cells. The virus was subjected to four successive plaque purifications. One plaque isolate was further amplified in primary CEF cells and a stock virus, designated as TROVAC, established.

NYVAC, ALVAC and TROVAC viral vectors and their derivatives were propagated as described previously (Piccini et al., 1987; Taylor et al., 1988a,b). Vero cells and chick embryo fibroblasts (CEF) were propagated as described previously (Taylor et al., 1988a,b). P815 murine mastocytoma cells (H-2^d) were obtained from ATCC (#TIB64) and maintained in Eagles MEM supplemented with 10% fetal bovine serum CFBS and 100 IU/ml penicillin and 100 μg streptomycin per ml.

Mice

Female BALB/cJ (H-2^d) mice were purchased from The Jackson Laboratories (Bar Harbor, Me.) and maintained on mouse chow and water ad libitum. All mice were used between the ages of 6 and 15 weeks of age.

Media

Assay Medium for immunological assays was comprised of RPMI 1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate), 5×10^{-5}M 2-mercaptoethanol, 100 IU penicillin per ml, and 100 μg/ml streptomycin. Stim Medium was comprised of Eagle's Minimum Essential Medium supplemented with 10% FBS, 4 mM L-glutamine, 10^{-4}M 2-mercaptoethanol, 100 IU penicillin per ml, and 100 μg streptomycin per ml.

Radioimmunoprecipitation Analysis

Cell monolayers were infected at 10 PFU/cell in modified Eagle's methionine-free medium (MEM met-). At 2 hours post-infection, 20 uCi/ml of [^{35}S]-methionine were added in MEM (-met) containing 2% dialysed fetal bovine serum (Flow). Cells were harvested at 15 hrs post-infection by resuspending them in lysis buffer (150 mM NaCl, 1 mM EDTA pH 8, 10 mM Tris-HCl (pH 7.4), 0.2 mg/ml PMSF, 1% NP40, 0.01% Na Azide) and 50 μl aprotinin, scraped into eppendorf tubes and the lysate was clarified by spinning 20 minutes at 4° C. One third of the supernatant of a 60 mm diameter Petri dish was incubated with 1 μl normal human serum and 100 μl of protein A-Seraphose CL-4B (SPA) (Pharmacia) for 2 hours at room temperature. After spinning for 1 minute, the supernatant was incubated for 90 min at 4° C. with immune sera specifically recognizing HIV-1, HIV-2, or SIV proteins and 100 μl SPA.

The pellet was washed four times with lysis buffer and two times with lithium chloride/urea buffer (0.2M LiCl, 2M urea, 10 mM Tris-HCl pH 8) and the precipitated proteins were dissolved in 60 μl Laemmli buffer (Laemmli, 1970). After heating for 5 minutes at 100° C. and spinning for 1 minute, proteins were resolved on a SDS 10% polyacrylamide gel and fluorographed.

Inoculations

Mice were intravenously inoculated with 5×10^7 plaque forming units (PFU) in 0.1 ml of phosphate-buffered saline via the lateral tail vein.

Spleen Cell Preparations

Following euthanasia by cervical dislocation, the spleens of mice were aseptically transferred to a sterile plastic bag containing Hank's Balanced Salt Solution. Individual spleens or pooled spleens from a single experimental group were processed to single cell suspensions by a 1 minute cycle in a Stomacher blender. The spleen cell suspensions were washed several times in either Assay Medium or Stim Medium, as appropriate. The spleen cells were enumerated by the use of a Coulter Counter or by trypan blue dye exclusion using a hemacytometer and microscope.

Sera

Mice were lightly anesthetized with ether and blood was collected from the retroorbital plexus. Blood from mice comprising an experimental group was pooled and allowed to clot. The serum was collected and stored at −70° C. until use.

In Vitro Stimulation for the Generation of Secondary Cytotoxic T Lymphocytes (CTL)

The pooled spleen cells from the various experimental groups (responders) were diluted to 5×10^6/ml in Stim Medium. The spleen cells from syngeneic, naive mice (stimulators) were diluted to 1×10^7 cells per ml and infected for 1 hour in tissue culture medium containing 2% FBS at 37° C. with the appropriate poxvirus at an m.o.i. of 25 PFU/cell. Following infection, the stimulator cells were washed several times in Stim Medium and diluted to 1×10^6 cells per ml with Stim Medium. Five mls of stimulator cells and 5 mls of responder cells were added to a 25 cm^3 tissue culture flask and incubated upright at 37° C., in 5% CO_2 for 5 days. On the day of the assay, the spleen cells were washed several times in Assay Medium and counted on a hemacytometer in trypan blue with the use of a microscope.

Target Cell Preparation

For poxvirus specific CTL activity, tissue culture cells were infected overnight by incubation at 1×10^7 cells per ml in tissue culture medium containing 2% FBS at an m.o.i. of 25 PFU/cell for 1 hour at 37° C. Following incubation, the cells were diluted to between 1-2×10^6 cells per ml with tissue culture medium containing 10% FBS and further incubated at 37° C., in 5% CO_2 until use. For HIV specific CTL activity, tissue culture cells were incubated overnight with 20 μg/ml of peptide HBX2 (American Biotechnologies, Cambridge, Mass.), SF2 (American Biotechnologies, Cambridge, Mass.) or MN, (American Biotechnologies, Cambridge, Mass.) corresponding to the V3 loop region of gp120 of HIV-1 isolates III_B, SF2, and MN, respectively. On the day of the assay, the targets were washed several times by centrifugation in Assay Medium. After the final wash, the cells were resuspended in approximately 100 μCi of $Na_2{}^{51}CrO_4$ (^{51}Cr). Following incubation at 37° C. for 1 hr, the cells were washed at least 3 times in Assay Medium by centrifugation, counted on a hemacytometer, and diluted to 1×10^5/ml in Assay Medium.

Cytotoxicity Assays

For primary CTL assays, freshly prepared spleen cells were diluted with Assay Medium to 1×10^7 cells per ml. For secondary CTL assays (following either in vivo inoculation or in vitro stimulation), the spleen cells were diluted to 2×10^6/ml in Assay Medium. One tenth ml of spleen cell suspension was added to ^{51}Cr labelled target cells in the wells of a 96 well, round-bottom microtiter plate (EXP). In most cases, the spleen cells being assayed for primary CTL activity were further 2-fold diluted in the wells of the microtiter plate prior to the addition of the target cells. As a measure of spontaneous release of ^{51}Cr (SR), target cells were incubated in only Assay Medium. To determine the maximum release of ^{51}Cr (MAX), target cells were deliberately lysed at the beginning of the assay by adding 0.1 ml of 10% sodium dodecyl sulfate to the appropriate wells. To initiate the assay, the microtiter plates were centrifuged at

200×g for 2 min and incubated for 4 or 5 hrs at 37° C., in 5% CO_2. Following incubation, the culture supernatants of each well were collected using the Skatron Supernatant Collection System. Released ^{51}Cr was determined by a Beckman 5500B gamma counter. The percent specific cytotoxicity was determined from the counts by the following formula:

$$\% \text{ CYTOTOXICITY}=(\text{EXP-MAX})/(\text{MAX-SR})\times 100$$

Depletion of T Helper Cells and Cytotoxic T Lymphocytes Using Monoclonal Anti-CD4 and Monoclonal Anti-CD8

Spleen cell suspensions were diluted to a density of 10^7/ml in cytotoxicity medium (RPMI 1640 containing 0.2% BSA and 5 mM HEPES) containing a 1:5 dilution of anti-CD4 (monoclonal antibody 172.4) or a 1:200 dilution of anti-CD8 (monoclonal antibody anti-Lyt 2.2) and a 1:16 dilution of Cedar Lane Low-Tox rabbit complement. Appropriate controls for the single components (complement, anti-CD4, anti-CD8) were included.

Anti-HIV-1 gp160 ELISA

The wells of ELISA plates (Immulon II) were coated overnight at 4° C. with 100 ng of purified HIV-1 gp160 (Immuno) in carbonate buffer, pH 9.6. The plates were then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST). The plates were then blocked for 2 hrs at 37° C. with PBST containing 1% bovine serum albumin (BSA). After washing with PBST, sera were initially diluted 1:20 with PBST containing 0.1% BSA (dilution buffer). The sera were further 2-fold serially diluted in the wells of the ELISA plate. The plates were incubated at 37° C. for 2 hrs and washed with PBST. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins (DAKO) was diluted 1:2000 in dilution buffer and added to the wells of the ELISA plated and incubated at 37° C. for 1 hour. After washing with PBST, OPD (o-phenylenediamine dihydrochloride) in substrate buffer was added and the color was allowed to develop at ambient temperature for about 20 minutes. The reaction was extinguished by the addition of 2.5M H_2SO_4. The absorbance at 490 nm was determined on a Bio-Tek EL-309 ELISA reader. The serum endpoint was defined as the reciprocal of the dilution giving an absorbance value of 1.0.

Lymphocyte Proliferation Assays

Single cell suspensions of the spleen cells of individual mice were diluted to 2×10^6/ml in Assay Medium and 0.1 ml were added to the wells of 96 well, flat-bottom microtiter plates containing Assay Medium alone, 1, 5, or 10 μg of HIV-1 peptide T1, 1, 5, or 10 μg of HIV-1 peptide T2, and 1 or 10 μg of purified HIV-1 gp160 (Immuno). The cells were incubated for 5 days at 37° C., in 5% CO_2. To each well was added 1.0 μCi of [^{3}H]-thymidine for the final 6 hrs of incubation and then harvested onto Beckman Ready Filters using a Cambridge PHD cell harvester. The filter disks were dry-counted in a liquid scintillation counter.

$$\text{STIMULATION INDEX}=\text{CPMs}_{EXP}/\text{CPMs}_{MEDIUM}$$

EXAMPLE 1

ATTENUATED VACCINIA VACCINE STRAIN NYVAC

To develop a new vaccinia vaccine strain, NYVAC (vP866), the Copenhagen vaccine strain of vaccinia virus

was modified by the deletion of six nonessential regions of the genome encoding known or potential virulence factors. The sequential deletions are detailed below. All designations of vaccinia restriction fragments, open reading frames and nucleotide positions are based on the terminology reported in Goebel et al. (1990a,b).

The deletion loci were also engineered as recipient loci for the insertion of foreign genes.

The regions sequentially deleted in NYVAC are listed below. Also listed are the abbreviations and open reading frame designations for the deleted regions (Goebel et al., 1990a,b) and the designation of the vaccinia recombinant (vP) containing all deletions through the deletion specified:

(1) thymidine kinase gene (TK; J2R) vP410;

(2) hemorrhagic region (u; B13R+B14R) vP553;

(3) A type inclusion body region (ATI; A26L) vP618;

(4) hemagglutinin gene (HA; A56R) vP723;

(5) host range gene region (C7L–K1L) vP804; and

(6) large subunit, ribonucleotide reductase (I4L) vP866 (NYVAC).

As described in following Examples, any or any combination of these regions can be a site either alone or in combination with other sites for inserting exogenous DNA from an immunodeficiency virus, immunodeficiency viruses or from such virus or viruses and other exogenous DNA to obtain a useful recombinant.

(1) Construction of Plasmid pSD460 for Deletion of Thymidine Kinase Gene (J2R)

Referring now to FIG. 1, plasmid pSD406 contains vaccinia HindIII J (pos. 83359–88377) cloned into pUC8. pSD406 was cut with HindIII and PvuII, and the 1.7 kb fragment from the left side of HindIII J cloned into pUC8 cut with HindIII/SmaI, forming pSD447. pSD447 contains the entire gene for J2R (pos. 83855–84385). The initiation codon is contained within an NlaIII site and the termination codon is contained within an SspI site. Direction of transcription is indicated by an arrow in FIG. 1.

To obtain a left flanking arm, a 0.8 kb HindIII/EcoRI fragment was isolated from pSD447, then digested with NlaIII and a 0.5 kb HindIII/NlaIII fragment isolated. Annealed synthetic oligonucleotides MPSYN43/MPSYN44 (SEQ ID NO:1/SEQ ID NO:2)

```
MPSYN43 5'      TAATTAACTAGCTACCCGGG    3'
MPSYN44 3' GTACATTAATTGATCGATGGGCCCTTAA 5'
           NlaIII                 EcoRI
```

were ligated with the 0.5 kb HindIII/NlaIII fragment into pUC18 vector plasmid cut with HindIII/EcoRI, generating plasmid pSD449.

To obtain a restriction fragment containing a vaccinia right flanking arm and pUC vector sequences, pSD447 was cut with SspI (partial) within vaccinia sequences and HindIII at the pUC/vaccinia junction, and a 2.9 kb vector fragment isolated. This vector fragment was ligated with annealed synthetic oligonucleotides MPSYN45/MPSYN46 (SEQ ID NO:3/SEQ ID NO:4)

```
           HindIII     SmaI
MPSYN45 5'  AGCTTCCCGGGTAAGTAATACGTCAAGGAGAAAACGAA
MPSYN46 3'       AGGGCCCATTCATTATGCAGTTCCTCTTTTGCTT

                          NotI                     SspI
           ACGATCTGTAGTTAGCGGCCGCCTAATTAACTAAT  3'MPSYN45
           TGCTAGACATCAATCGCCGGCGGATTAATTGATTA  5'MPSYN46
```

generating pSD459.

To combine the left and right flanking arms into one plasmid, a 0.5 kb HindIII/SmaI fragment was isolated from pSD449 and ligated with pSD459 vector plasmid cut with HindIII/SmaI, generating plasmid pSD460. pSD460 was used as donor plasmid for recombination with wild type parental vaccinia virus Copenhagen strain VC-2. ^{32}P labelled probe was synthesized by primer extension using MPSYN45 (SEQ ID NO:3) as template and the complementary 20mer oligonucleotide MPSYN47 (SEQ ID NO:5) (5' TTAGTTAATTAGGCGGCCGC 3') as primer. Recombinant virus vP410 was identified by plaque hybridization.

(2) Construction of Plasmid pSD486 for Deletion of Hemorrhagic Region (B13R+B14R)

Referring now to FIG. 2, plasmid pSD419 contains vaccinia SalI G (pos. 160,744–173,351) cloned into pUC8. pSD422 contains the contiguous vaccinia SalI fragment to the right, SalI J (pos. 173,351–182,746) cloned into pUC8. To construct a plasmid deleted for the hemorrhagic region, u, B13R–B14R (pos. 172,549–173,552), pSD419 was used as the source for the left flanking arm and pSD422 was used as the source of the right flanking arm. The direction of transcription for the u region is indicated by an arrow in FIG. 2.

To remove unwanted sequences from pSD419, sequences to the left of the NcoI site (pos. 172,253) were removed by digestion of pSD419 with NcoI/SmaI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation generating plasmid pSD476. A vaccinia right flanking arm was obtained by digestion of pSD422 with HpaI at the termination codon of B14R and by digestion with NruI 0.3 kb to the right. This 0.3 kb fragment was isolated and ligated with a 3.4 kb HincII vector fragment isolated from pSD476, generating plasmid pSD477. The location of the partial deletion of the vaccinia u region in pSD477 is indicated by a triangle. The remaining B13R coding sequences in pSD477 were removed by digestion with ClaI/HpaI, and the resulting vector fragment was ligated with annealed synthetic oligonucleotides SD22mer/SD20mer (SEQ ID NO:6/SEQ ID NO:7)

```
          ClaI            BamHI  HpaI
SD22mer 5' CGATTACTATGAAGGATCCGTT 3'
SD20mer 3'   TAATGATACTTCCTAGGCAA 5'
```

generating pSD479. pSD479 contains an initiation codon (underlined) followed by a BamHI site. To place *E. coli* Beta-galactosidase in the B13–B14 (u) deletion locus under the control of the u promoter, a 3.2 kb BamHI fragment containing the Beta-galactosidase gene (Shapira et al., 1983) was inserted into the BamHI site of pSD479, generating pSD479BG. pSD479BG was used as donor plasmid for recombination with vaccinia virus vP410. Recombinant vaccinia virus vP533 was isolated as a blue plaque in the presence of chromogenic substrate X-gal. In vP533 the B13R–B14R region is deleted and is replaced by Beta-galactosidase.

To remove Beta-galactosidase sequences from vP533, plasmid pSD486, a derivative of pSD477 containing a polylinker region but no initiation codon at the u deletion junction, was utilized. First the ClaI/HpaI vector fragment from pSD477 referred to above was ligated with annealed synthetic oligonucleotides SD42mer/SD40mer (SEQ ID NO:8/SEQ ID NO:9)

```
          ClaI           SacI          XhoI          HpaI
SD42mer 5' CGATTACTAGATCTGAGCTCCCCCGGGCTCGAGGGATCCGTT  3'
SD40mer 3'   TAATGATCTAGACTCGAGGGGGCCCGAGCTCCCTAGGCAA  5'
                  BglII          SmaI          BamHI
```

generating plasmid pSD478. Next the EcoRI site at the pUC/vaccinia junction was destroyed by digestion of pSD478 with EcoRI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation, generating plasmid pSD478E⁻. pSD478E⁻ was digested with BamHI and HpaI and ligated with annealed synthetic oligonucleotides HEM5/HEM6 (SEQ ID NO:10/SEQ ID NO:11)

```
         BamHI EcoRI   HpaI
HEM5 5'  GATCCGAATTCTAGCT 3'
HEM6 3'      GCTTAAGATCGA 5'
```

generating plasmid pSD486. pSD486 was used as donor plasmid for recombination with recombinant vaccinia virus vP533, generating vP553, which was isolated as a clear plaque in the presence of X-gal.

(3) Construction of Plasmid pMP494Δ for Deletion of ATI Region (A26L)

Referring now to FIG. 3, pSD414 contains SalI B cloned into pUC8. To remove unwanted DNA sequences to the left of the A26L region, pSD414 was cut with XbaI within vaccinia sequences (pos. 137,079) and with HindIII at the pUC/vaccinia junction, then blunt ended with Klenow fragment of *E. coli* polymerase and ligated, resulting in plasmid pSD483. To remove unwanted vaccinia DNA sequences to the right of the A26L region, pSD483 was cut with EcoRI (pos. 140,665 and at the pUC/vaccinia junction) and ligated, forming plasmid pSD484. To remove the A26L coding region, pSD484 was cut with NdeI (partial) slightly upstream from the A26L ORF (pos. 139,004) and with HpaI (pos. 137,889) slightly downstream from the A26L ORF. The 5.2 kb vector fragment was isolated and ligated with annealed synthetic oligonucleotides ATI3/ATI4 (SEQ ID NO:12/SEQ ID NO:13)

```
        NdeI
ATI3 5' TATGAGTAACTTAACTCTTTTGTTAATTAAAAGTATATTCAAAAAATAAGT
ATI4 3'    ACTCATTGAATTGAGAAAACAATTAATTTTCATATAAGTTTTTTATTCA

         BglII   EcORI  HpaI
TATATAAATAGATCTGAATTCGTT 3' ATI3
ATATATTTATCTAGACTTAAGCAA 5' ATI4
```

reconstructing the region upstream from A26L and replacing the A26L ORF with a short polylinker region containing the restriction sites BglII. EcoRI and HpaI. as indicated above. The resulting plasmid was designated pSD485. Since the BglII and EcoRI sites in the polylinker region of pSD485 are not unique. unwanted BglII and EcoRI sites were removed from plasmid pSD483 (described above) by digestion with BglII (pos. 140.136) and with EcoRI at the pUC/vaccinia junction. followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation. The resulting plasmid was designated pSD489. The 1.8 kb ClaI (pos. 137.198)/EcoRV (pos. 139.048) fragment from pSD489 containing the A26L ORF was replaced with the corresponding 0.7 kb polylinker-containing ClaI/EcoRV fragment from pSD485. generating pSD492. The BglII and EcoRI sites in the polylinker region of pSD492 are unique.

A 3.3 kb BglII cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Perkus et al., 1990) was inserted into the BglII site of pSD492. forming pSD493KBG. Plasmid pSD493KBG was used in recombination with rescuing virus vP553. Recombinant vaccinia virus. vP581. containing Beta-galactosidase in the A26L deletion region. was isolated as a blue plaque in the presence of X-gal.

To generate a plasmid for the removal of Beta-galactosidase sequences from vaccinia recombinant virus vP581. the polylinker region of plasmid pSD492 was deleted by mutagenesis (Mandecki. 1986) using synthetic oligonucleotide MPSYN177 (SEQ ID NO:14) (5' AAAATGGGCGTGGATTGTTAACTTTATATAACTTATTTTTTGAATATAC 3'). In the resulting plasmid. pMP494Δ. vaccinia DNA encompassing positions [137.889–138.937]. including the entire A26L ORF is deleted. Recombination between the pMP494Δ and the Beta-galactosidase containing vaccinia recombinant. vP581. resulted in vaccinia deletion mutant vP618. which was isolated as a clear plaque in the presence of X-gal.

(4) Construction of Plasmid pSD467 for Deletion of Hemagglutinin Gene (A56R)

Referring now to FIG. 4. vaccinia SalI G restriction fragment (pos. 160.744–173.351) crosses the HindIII A/B junction (pos. 162.539). pSD419 contains vaccinia SalI G cloned into pUC8. The direction of transcription for the hemagglutinin (HA) gene is indicated by an arrow in FIG. 4. Vaccinia sequences derived from HindIII B were removed by digestion of pSD419 with HindIII within vaccinia sequences and at the pUC/vaccinia junction followed by ligation. The resulting plasmid. pSD456. contains the HA gene. A56R. flanked by 0.4 kb of vaccinia sequences to the left and 0.4 kb of vaccinia sequences to the right. A56R coding sequences were removed by cutting pSD456 with RsaI (partial; pos. 161.090) upstream from A56R coding sequences. and with EagI (pos. 162.054) near the end of the gene. The 3.6 kb RsaI/EagI vector fragment from pSD456 was isolated and ligated with annealed synthetic oligonucleotides MPSYN59 (SEQ ID NO:15). MPSY62 (SEQ ID NO:16). MPSYN60 (SEQ ID NO:17). and MPSYN 61 (SEQ ID NO:18)

```
           RsaI
MPSYN59 5' ACACGAATGATTTTCTAAAGTATTTGGAAAGTTTTATAGGTAGTTGATAGA-
MPSYN62 3' TGTGCTTACTAAAAGATTTCATAAACCTTTCAAAATATCCATCAACTATCT   5'

MPSYN59      -ACAAAATACATAATTT 3'

                                                      BglII
MPSYN60 5'                    TGTAAAAATAAATCACTTTTTATACTAAGATCT-
MPSYN61 3' TGTTTTATGTATTAAAACATTTTTATTTAGTGAAAAATATGATTCTAGA-

           SmaI  PstI   EagI
MPSYN60 -CCCGGGCTGCAGC     3'
MPSYN61 -GGGCCCGACGTCGCCGG 5'
```

reconstructing the DNA sequences upstream from the A56R ORF and replacing the A56R ORF with a polylinker region as indicated above. The resulting plasmid is pSD466. The vaccinia deletion in pSD466 encompasses positions [161. 185–162.053]. The site of the deletion in pSD466 is indicated by a triangle in FIG. 4.

A 3.2 kb BglII/BamHI (partial) cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Guo et al., 1989) was inserted into the BglII site of pSD466. forming pSD466KBG. Plasmid pSD466KBG was used in recombination with rescuing virus vP618. Recombinant vaccinia virus. vP708. containing Beta-galactosidase in the A56R deletion. was isolated as a blue plaque in the presence of X-gal.

Beta-galactosidase sequences were deleted from vP708 using donor plasmid pSD467. pSD467 is identical to pSD466. except that EcoRI. SmaI and BamHI sites were removed from the pUC/vaccinia junction by digestion of pSD466 with EcoRI/BamHI followed by blunt ending with Klenow fragment of *E. coli* polymerase and ligation. Recombination between vP708 and pSD467 resulted in recombinant vaccinia deletion mutant. vP723. which was isolated as a clear plaque in the presence of X-gal.

(5) Construction of Plasmid pMPCSK1Δ for Deletion of Open Reading Frames [C7L–K1L]

Referring now to FIG. 5. the following vaccinia clones were utilized in the construction of pMPCSK1Δ. pSD420 is SalI H cloned into pUC8. pSD435 is KpnI F cloned into pUC18. pSD435 was cut with SphI and religated. forming pSD451. In pSD451. DNA sequences to the left of the SphI

site (pos. 27,416) in HindIII M are removed (Perkus et al., 1990). pSD409 is HindIII M cloned into pUC8.

To provide a substrate for the deletion of the [C7L–K1L] gene cluster from vaccinia, *E. coli* Beta-galactosidase was first inserted into the vaccinia M2L deletion locus (Guo et al., 1990) as follows. To eliminate the BglII site in pSD409, the plasmid was cut with BglII in vaccinia sequences (pos. 28,212) and with BamHI at the pUC/vaccinia junction, then ligated to form plasmid pMP409B. pMP409B was cut at the unique SpHI site (pos. 27,416). M2L coding sequences were removed by mutagenesis (Guo et al., 1990; Mandecki, 1986) using synthetic oligonucleotide.

and the Beta-galactosidase containing vaccinia recombinant, vP784, resulted in vaccinia deletion mutant, vP804, which was isolated as a clear plaque in the presence of X-gal.

(6) Construction of Plasmid pSD548 for Deletion of Large Subunit, Ribonucleotide Reductase (I4L)

Referring now to FIG. 6, plasmid pSD405 contains vaccinia HindIII I (pos. 63,875–70,367) cloned in pUC8. pSD405 was digested with EcoRV within vaccinia sequences (pos. 67,933) and with SmaI at the pUC/vaccinia junction, and ligated, forming plasmid pSD518. pSD518 was used as the source of all the vaccinia restriction fragments used in the construction of pSD548.

```
                                                          BglII
MPSYN82  (SEQ ID NO:19) 5' TTTCTGTATATTTGCACCAATTTAGATCTTACTC
                           AAAATATGTAACAATA 3'
```

The resulting plasmid, pMP409D, contains a unique BglII site inserted into the M2L deletion locus as indicated above. A 3.2 kb BamHI (partial)/BglII cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the 11 kDa promoter (Bertholet et al., 1985) was inserted into pMP409D cut with BglII. The resulting plasmid, pMP409DBG (Guo et al., 1990), was used as donor plasmid for recombination with rescuing vaccinia virus vP723. Recombinant vaccinia virus, vP784, containing Beta-galactosidase inserted into the M2L deletion locus, was isolated as a blue plaque in the presence of X-gal.

A plasmid deleted for vaccinia genes [C7L–K1L] was assembled in pUC8 cut with SmaI, HindIII and blunt ended with Klenow fragment of *E. coli* polymerase. The left flanking arm consisting of vaccinia HindIII C sequences was obtained by digestion of pSD420 with XbaI (pos. 18,628) followed by blunt ending with Klenow fragment of *E. coli* polymerase and digestion with BglII (pos. 19,706). The right flanking arm consisting of vaccinia HindIII K sequences was obtained by digestion of pSD451 with BglII (pos. 29,062) and EcoRV (pos. 29,778). The resulting plasmid, pMP581CK is deleted for vaccinia sequences between the BglII site (pos. 19,706) in HindIII C and the BglII site (pos. 29,062) in HindIII K. The site of the deletion of vaccinia sequences in plasmid pMP581CK is indicated by a triangle in FIG. 5.

The vaccinia I4L gene extends from position 67,371–65,059. Direction of transcription for I4L is indicated by an arrow in FIG. 6. To obtain a vector plasmid fragment deleted for a portion of the I4L coding sequences, pSD518 was digested with BamHI (pos. 65,381) and HpaI (pos. 67,001) and blunt ended using Klenow fragment of *E. coli* polymerase. This 4.8 kb vector fragment was ligated with a 3.2 kb SmaI cassette containing the *E. coli* Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985; Perkus et al., 1990), resulting in plasmid pSD524KBG. pSD524KBG was used as donor plasmid for recombination with vaccinia virus vP804. Recombinant vaccinia virus, vP855, containing Beta-galactosidase in a partial deletion of the I4L gene, was isolated as a blue plaque in the presence of X-gal.

To delete Beta-galactosidase and the remainder of the I4L ORF from vP855, deletion plasmid pSD548 was constructed. The left and right vaccinia flanking arms were assembled separately in pUC8 as detailed below and presented schematically in FIG. 6.

To construct a vector plasmid to accept the left vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and ligated with annealed synthetic oligonucleotides 518A1/518A2 (SEQ ID NO:21/SEQ ID NO:22)

```
          BamHI    RsaI
518A1 5' GATCCTGAGTACTTTGTAATATAATGATATATATTTTCACTTTATCTCAT
518A2 3'         GACTCATGAAACATTATATTACTATATATAAAAGTGAAATAGAGTA

         BglII          EcoRI
         TTGAGAATAAAAAGATCTTAGG         3' 518A1
         AACTCTTATTTTTCTAGAATCCTTAA 5' 518A2
```

To remove excess DNA at the vaccinia deletion junction, plasmid pMP581CK, was cut at the NcoI sites within vaccinia sequences (pos. 18,811; 19,655), treated with Bal-31 exonuclease and subjected to mutagenesis (Mandecki, 1986) using synthetic oligonucleotide MPSYN233 (SEQ ID NO:20) 5' TGTCATTTAACACTATACTCATATTAATAAAAATAATATTTATT 3'. The resulting plasmid, pMPCSK1Δ, is deleted for vaccinia sequences positions 18,805–29,108, encompassing 12 vaccinia open reading frames [C7L–K1L]. Recombination between pMPCSK1Δ

forming plasmid pSD531. pSD531 was cut with RsaI (partial) and BamHI and a 2.7 kb vector fragment isolated. pSD518 was cut with BglII (pos. 64,459)/ RsaI (pos. 64,994) and a 0.5 kb fragment isolated. The two fragments were ligated together, forming pSD537, which contains the complete vaccinia flanking arm left of the I4L coding sequences.

To construct a vector plasmid to accept the right vaccinia flanking arm, pUC8 was cut with BamHI/EcoRI and ligated with annealed synthetic oligonucleotides 518B1/518B2 (SEQ ID NO:23/SEQ ID NO:24)

```
           BamHI  BglII   SmaI
518B1 5'  GATCCAGATCTCCCGGGAAAAAAATTATTTAACTTTTCATTAATAGGGATTT
```

-continued

```
518B2 3'   GTCTAGAGGGCCCTTTTTTTAATAAATTGAAAAGTAATTATCCCTAAA

                         RsaI       EcoRI
           GACGTATGTAGCGTACTAGG             3' 518B1
           CTGCATACTACGCATGATCCTTAA         5' 518B2
```

forming plasmid pSD532. pSD532 was cut with RsaI (partial)/EcoRI and a 2.7 kb vector fragment isolated. pSD518 was cut with RsaI within vaccinia sequences (pos. 67,436) and EcoRI at the vaccinia/pUC junction, and a 0.6 kb fragment isolated. The two fragments were ligated together, forming pSD538, which contains the complete vaccinia flanking arm to the right of I4L coding sequences.

The right vaccinia flanking arm was isolated as a 0.6 kb EcoRI/BglII fragment from pSD538 and ligated into pSD537 vector plasmid cut with EcoRI/BglII. In the resulting plasmid, pSD539, the I4L ORF (pos. 65,047–67,386) is replaced by a polylinker region, which is flanked by 0.6 kb vaccinia DNA to the left and 0.6 kb vaccinia DNA to the right, all in a pUC background. The site of deletion within vaccinia sequences is indicated by a triangle in FIG. 6. To avoid possible recombination of Beta-galactosidase sequences in the pUC-derived portion of pSD539 with Beta-galactosidase sequences in recombinant vaccinia virus vP855, the vaccinia I4L deletion cassette was moved from pSD539 into pRC11, a pUC derivative from which all Beta-galactosidase sequences have been removed and replaced with a polylinker region (Colinas et al., 1990). pSD539 was cut with EcoRI/PstI and the 1.2 kb fragment isolated. This fragment was ligated into pRC11 cut with EcoRI/PstI (2.35 kb) forming pSD548. Recombination between pSD548 and the Beta-galactosidase containing vaccinia recombinant, vP855, resulted in vaccinia deletion mutant vP866, which was isolated as a clear plaque in the presence of X-gal.

DNA from recombinant vaccinia virus vP866 was analyzed by restriction digests followed by electrophoresis on an agarose gel. The restriction patterns were as expected. Polymerase chain reactions (PCR) (Engelke et al., 1988) using vP866 as template and primers flanking the six deletion loci detailed above produced DNA fragments of the expected sizes. Sequence analysis of the PCR generated fragments around the areas of the deletion junctions confirmed that the junctions were as expected. Recombinant vaccinia virus vP866, containing the six engineered deletions as described above, was designated vaccinia vaccine strain "NYVAC."

EXAMPLE 2

EXPRESSION OF HIV GENE PRODUCTS BY HOST-RESTRICTED POXVIRUSES

This Example describes the generation of host-restricted poxviruses that express HIV gene products. The vectors employed are NYVAC, ALVAC and TROVAC.

ALVAC and NYVAC Recombinants Containing the HIV-1 (IIIB) V3 Loop and Epitope 88

A 150 bp fragment encompassing the V3 loop (amino acids 299–344; Javeherian et al., 1989; La Rosa et al., 1990) of HIV-1 (IIIB) was derived by PCR using oligonucleotides HIV3BL5 (SEQ ID NO:25) (5'-ATGGTAGAAATTAATTGTAC-3') and HIV3BL3 (SEQ ID NO:26) (5'-ATCATCGAATTCAAGCTTATTATTTTGCTCTACTAATGTTAC-3') with pHXB.2D (III) as template (provided by Dr. R. C. Gallo, NCI-NIH, Bethesda, Md.). Oligonucleotides HIV88A (SEQ ID NO:27) (5'-ATGAATGTGACAGAAAATTTTAACATGTGGAAAAATGTAGAAATTAATTGTACAAGACCC-3') and HIV88B (SEQ ID NO:28) (5'-GGGTCTTGTACAATTAATTTCTACATTTTTCCACATGTTAAAATTTTCTGTCACATTCAT-3') were annealed together to produce a double-stranded fragment containing the HIV-1 epitope 88 (amino acids 95–105, Shaffermann et al., 1989). The 150 bp V3-containing PCR fragment containing the epitope and the 42 bp fragment containing the 88 epitope sequences were fused together by PCR by virtue of the existence of complementary sequences. The reactions were performed using oligonucleotides HIV88C (SEQ ID NO:29) (5'-AGTAATGTGACAGAAAATTTTAAC-3') and HIV3BL3. The 192 bp PCR-derived fragment contains the epitope 88 sequences fused upstream to the V3 loop sequences. A termination codon (TAA) was incorporated into oligonucleotide HIV3BL3P to terminate translation of the open reading frame and an initiation codon was incorporated into oligonucleotide HIV88C to serve as the start of translation to express the epitope 88/V3 loop fusion protein. Additionally, oligonucleotide HIV3BL3 was synthesized so that an EcoRI site existed at the 3'-end of the 192 bp PCR fragment.

The entomopoxvirus promoter, 42 kDa (early) was generated by PCR using oligonucleotides RG273 (SEQ ID NO:30) (5'-AGGCAAGCTTTCAAAAAAATATAAATGATTC-3') and RG274 (SEQ ID NO:31) (5'-TTTATATTGTAATTATATATTTTC-3') with plasmid, pAM12, as template. The 108 bp fragment containing the 42 kDa promoter was synthesized to contain a HindIII site at the 5'-end. The 42 kDa promoter containing segment was kinased and digested with HindIII prior to ligation to the epitope 88/V3 fragment digested with EcoRI and pRW831 digested with HindIII and EcoRI. The resultant plasmid was designated as pC5HIVL88. This plasmid was used in in vitro recombination assays with CPpp as rescue virus to generate vCP95. ALVAC recombinant, vCP95, contains the epitope 88/V3 loop in the de-ORFed C5 locus of CPpp.

The plasmid pC5HIVL88 was digested with HindIII and EcoRI to liberate a 300 bp fragment containing the epitope 88/V3 expression cassette described above. This fragment was excised from a LMP-agarose gel and isolated by phenol extraction (2X) and ether extraction (1X). The isolated fragment was blunt-ended using the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. The blunted fragment was ligated to pSD550VC digested with SmaI to yield plasmid pHIVL88VC. This plasmid was used with vP866 as the rescue virus to generate vP878. vP878 contains the epitope 88/V3 loop cassette in the de-ORFed I4L locus of vP866.

ALVAC- and NYVAC-Based Recombinants Expressing the HIV-1 (IIIB) Envelope Glycoproteins

An expression cassette composed of the HIV-1 (IIIB) env gene juxtaposed 3' to the vaccinia virus H6 promoter (Guo et al., 1989; Taylor et al., 1988a,b) was engineered for expression of gp160 from HIV-1 by the ALVAC and

NYVAC vectors. A 1.4 kb fragment was amplified from pHXB.2D (III) (provided by Dr. R. C. Gallo, NCI-NIH, Bethesda, Md.) using oligonucleotides HIV3B1 (SEQ ID NO:32) (5'-GTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTC-3') and HIV3B5 (SEQ ID NO:33) (5'-ATCATCTCTAGAATAAAAATTATAGCAAAATCCTTTC-3'). This fragment contains the 3' portion of the env gene. PCR amplification with these primers placed a vaccinia virus early transcription termination T5NT sequence motif following the coding sequence and removed the T5NT motif situated at position 6146 to 6152 (Ratner et al., 1985) without altering the amino acid sequence. This change (T to C) creates an EcoRI site (GAATTC) at this position. This 1.4 kb fragment was digested with EcoRI (5'-end) and XbaI (3'-end) and inserted into EcoRI and XbaI digested pBS-SK (Stratagene, La Jolla, Calif.). The resultant plasmid was designated as pBSHIVENV1.5. Nucleotide sequence analysis of this fragment demonstrated that the sequence was entirely correct except for a T to C transition at position 7048. This transition was corrected as follows: A 250 bp fragment was derived by PCR using oligonucleotides HIV3B1 (SEQ ID NO:32) (5'-GTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTC-3') and HIV3B17 (SEQ ID NO:34) (5'-TGCTACTCCTAATGGTTC-3') with pHXB.2D (III) as template. This fragment was digested with BglII and EcoRI. The fragment was inserted into pBSHIV3B1.5, digested with BglII and EcoRI and thus substituted for the region with the incorrect nucleotide to yield plasmid pBSHIV3B3P.

PCR was utilized to derive a 150 bp fragment containing the 5' portion of the env gene with oligonucleotides HIV3B9 (SEQ ID NO:35) (5'-CATATGCTTTAGCATCTGATG-3') and HIV3B10 (SEQ ID NO:36) (5'-ATGAAAGAGCAGAAGACAGTG-3') with pHXB.2D (III) as template. PCR was also used to generate a 128 bp fragment containing the vaccinia virus H6 promoter from pC3FGAG using oligonucleotides VV6K5P (SEQ ID NO:37) (5'-ATCATCGGTACCGATTCTTTATTCTATAC-3') and VVH63P (SEQ ID NO:38) (5'-TACGATACAAACTTAACGG-3'). Both fragments were digested with KpnI and the 150 bp fragment was kinased prior to co-insertion of these fragments into pBS-SK digested with KpnI. The resultant plasmid was designated as pBSH6HIV3B5P.

PCR was used to generate a 600 bp fragment from pHXB.2D (III) with oligonucleotides HIV3B2 (SEQ ID NO:39) (5'-GAATTACAGTAGAAGAATTCCCCTCCACAATTAAAAC-3') and HIV3B7 (SEQ ID NO:40) (5'-CAATAGATAATGATACTAC-3'). This fragment was digested with EcoRI and kinased. PCR was also used to derive a 500 bp fragment with the same template but with oligonucleotides HIV3B6 (SEQ ID NO:41) (5'-GTATTATATCAAGTTTATATAATAATGCATATTC-3') and HIV3B8 (SEQ ID NO:42) (5'-GTTGATGATCTGTAGTGC-3'). This fragment was digested with KpnI. These fragments together correspond to nucleotide 5878 to 6368 (Ratner et al., 1985). The engineering of these fragments with these primers also removes a T5NT sequence positioned at nucleotide 6322 to 6328 without altering the amino acid sequence. These two fragments were inserted into pBSHIV3B3P digested with KpnI and EcoRI. This plasmid was designated as pBSHIV3BP2768.

Plasmid pBSH6HIV3B5P was digested with KpnI to liberate a 360 bp fragment containing the H6 promoter and the 5' portion (150 bp) of the HIV-1 env gene. This KpnI fragment was ligated into pBSHIV3B3P2768 digested with KpnI to yield plasmid pBSHIV3BEII. A 2.8 kb fragment was derived from pBSHIV3BEII by digestion with XbaI followed by a partial KpnI digestion. This fragment was blunt-ended and inserted into SmaI digested pSD550. The plasmid pI4LH6HIV3B was generated and used in recombination experiments with vP866 as the rescue virus. This generated vP911 which contains the HIV-1 env gene in the I4L locus of the NYVAC genome.

To insert the HIV-1 env gene into an ALVAC vector, pBSHIV3BEAII was digested with NruI and XbaI. The derived 2.7 kb fragment was blunt-ended with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. This fragment contains the entire HIV-1 env gene juxtaposed 3' to the 3'-most 21 bp (to NruI site) of the vaccinia H6 promoter. This fragment was ligated to a 3.1 kb fragment derived by digestion of pRW838 with NruI and EcoRI with subsequent blunt-ending with Klenow. The pRW838 derived fragment contains the homologous arms derived from canarypox to direct the foreign gene to the C5 locus. It also contains the 5'-most 100 bp of the H6 promoter. Therefore, ligation of these fragments resulted in an insertion plasmid containing an expression cassette for the HIV-1 env gene and was designated pC5HIV3BE. This plasmid was used in in vitro recombination experiments with ALVAC as the rescue virus to generate vCP112.

NYVAC-Based Recombinants Expressing the HIV-1 (IIIB) gp120

The plasmid pBSHIV3BEAII was digested with EcoRI and XbaI to liberate a 4.3 kb fragment. This fragment contains the vaccinia virus H6 promoter linked to the HIV-1 env gene to nucleotide 6946 (Ratner et al., 1985). The 4.3 kb fragment was ligated to 300 bp EcoRI/XbaI digested PCR-derived fragment corresponding to the 3' portion of the gp120 coding sequence. The 300 bp PCR fragment was derived using oligonucleotides HIV1-120A (SEQ ID NO:43) (5'-ATCATCTCTAGAATAAAAATTATGGTTCAATTTTTACTACTTTTATATTATATATTTC-3') and HIV1-120B (SEQ ID NO:44) (5'-CAATAATCTTTAAGCAAATCCTC-3') with pHXB.2D (III) as template. The ligation of the 4.3 kb XbaI/EcoRI fragment and the 300 bp XbaI/EcoRI fragment yielded plasmid pBSHIVB120.

A 1.6 kb KpnI/XbaI fragment was derived from pBSHIVB120 by initially linearizing the plasmid with XbaI followed by a partial KpnI digestion. The 1.6 kb fragment was blunt-ended by treatment with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. This fragment was inserted into pSD541 digested with SmaI to yield pATIHIVB120. This plasmid was used in in vitro recombination experiments to generate vP921. This recombinant contains the portion of the HIV-1 env gene encoding gp120 in the ATI locus of NYVAC.

To determine the authenticity of the HIV-1 gene products expressed by vP911, vP921 and vCP112, immunoprecipitation analyses were performed.

Lysates derived from the infected cells were analyzed for HIV-1 env gene expression using pooled serum from HIV-1 seropositive individuals (obtained from Dr. Genoveffa Franchini, NCI-NIH, Bethesda, Md.). The sera was preadsorbed with vP866-infected Vero cells. The preadsorbed human sera was bound to protein A-sepharose in an overnight inoculation at 4° C. In some cases a monoclonal antiserum specific to gp120 (Dupont) was used as the primary serum and a rat anti-mouse as the second antibody. Following this incubation period, the material was washed 4

times with 1X Buffer A. Lysates precleared with normal human sera and protein A-Sepharose were then incubated overnight at 4° C. with the human sera from seropositive individuals bound to protein A-Sepharose. Following the overnight incubation period, the samples were washed four times with 1×Buffer A and 2×with $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol; 10% 2-mercaptoethanol) and boiling for 5 minutes. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984) fixed and treated with 1M - Na - salicylate for fluorography.

The results of immunoprecipitation using sera pooled from HIV-1 seropositive individuals showed specific precipitation of the gp120 and gp41 mature forms of the gp160 envelope glycoprotein from vP911 infected cell lysates. No such specific gene products can be detected in the parental virus (NYVAC; vP866) infected cell lysates. Specific precipitation of gp120 was also found in vP921 infected cell lysates.

Immunofluorescence analysis with the same sera illustrated that the gp160 and gp120 species expressed by vP911 and vP921, respectively, were present on the surface of infected cells.

Immunoprecipitation was also performed with vCP112 infected CEF cells. No HIV-specific polypeptides were precipitated with a monoclonal antibody directed against the gp120 extracellular moiety from cells infected with the ALVAC parental virus and uninfected CEF cells. Two HIV-specific polypeptides species were, however, precipitated from vCP112 infected cells. These species migrated with apparent mobilities of 160 kDa and 120 kDa, corresponding to the precursor env gene product and the mature extracellular form, respectively.

A Recombinant Vaccinia Virus Expressing HIV gp120 Elicits Primary HIV-specific Cytotoxic T Lymphocyte Activity

Following iv administration with 5×10^7 PFUs of vaccinia virus recombinants vP878, vP911, or vP921, or, as a control, with NYVAC, the vector, splenic CTL activity of BALB/c mice was assessed against syngeneic P815 cells which had been incubated overnight with peptide HBX2 (Table 1). Modest, but significant ($P<0.05$) primary CTL activity was generated in the spleens of mice administered vP921, expressing HIV gp120. No other recombinant vaccinia virus nor the vector was able to elicit primary HIV-specific CTL activity. This was not due to inadequate infection as each group of mice administered a vaccinia virus responded with primary vaccinia-specific CTL activity. Control, unimmunized mice responded to neither target.

Recombinant Poxviruses Expressing HIV env Peptides Generate HIV-Specific Memory Cytotoxic T Lymphocytes

At least one month following a single inoculation with one of the recombinant vaccinia viruses, mouse spleen cells were stimulated in vitro with syngeneic, naive spleen cells previously infected with NYVAC or with each of the HIV recombinant vaccinia viruses (Table 2). Strong HIV-specific CTL activity was detected only in the spleen cell cultures of mice immunized with vP878, vP911, and vP921 which were restimulated in vitro by cells infected with one of the same vaccinia virus HIV recombinants (vP878, vP911, or vP921). The vaccinia virus recombinants expressing HIV gp120 or gp160 were better able to generate memory CTLs than the vaccinia virus recombinant expressing only the V3 loop fused to the 88 epitope. HIV-specific memory CTL activity could not be elicited from unimmunized control or NYVAC immunized spleen cells. The absence of HIV-specific CTL activity from vector immunized mice could not be attributed to poor immunization since vaccinia-specific memory CTL activity was apparent after in vitro stimulation with spleen cells infected with any of the vaccinia viruses used.

In a similar study, the ability of a canarypox recombinant expressing the V3 loop region fused to the 88 epitope (vCP95) to generate HIV-specific memory CTLs was examined (Table 3). Three weeks following a single inoculation of 10^8 PFUs of vCP95 or the canarypox vector, CPpp, HIV-specific memory CTL responses were compared to that elicited by the recombinant vaccinia virus analog, vP878. Vaccinia and canarypox CTL responses were included as controls for proper immunization. Only spleen cells from vP878 and vCP95 immunized mice produced HIV-specific memory CTL activity which could be stimulated by vP878. The inability of vCP95 to stimulate existing memory CTLs to functional cytolytic CTLs may have been related to the in vitro conditions employed which were maximized based upon the use of vaccinia virus recombinants. Nonetheless, vCP95 was fully capable of generating significant HIV-specific memory CTLs in the spleens of immunized mice.

Characterization of the In Vitro Stimulated Cytotoxic Cells

It is conceivable that the cells mediating cytotoxicity against the HIV peptide-pulsed target cells represent a population of nonspecific effector cells unrelated to CTLs, such as natural killer cells. To test this, the spleen cells of mice immunized with vP921 and restimulated in vitro with vP921 infected spleen cells were depleted of T-lymphocytes bearing surface antigens characteristic of T helper lymphocytes (CD4) or of cytotoxic T lymphocytes (CD8) and assayed against V3 loop peptide pulsed target cells (Table 4). As before, only vP921 immunized mice generated memory HIV-specific CTL activity which could be stimulated in vitro with vP921 infected syngeneic spleen cells. Although the complement preparation (C') and the monoclonal anti-CD4 and anti-CD8 produced some toxic effects, only the cultures depleted of CD8-bearing cells (anti-CD8+C') were also depleted of HIV-specific cytotoxic effector cells. Thus, the cells mediating cytolytic activity against the HIV peptide-pulsed target cells possessed CD8 antigens on their cell membranes, a characteristic of MHC class I restricted CTLs.

Specificity of CTL Antigen Receptor Recognition of the V3 Loop Region of HIV gp120

T lymphocyte antigen receptors are exquisitely sensitive to small alterations in the primary amino acid sequence of the epitope fragment. The V3 loop region of HIV gp120 is hypervariable and differs immunologically among HIV isolates. The hypervariability resides in substitutions and additions of only a few amino acids. To examine the specificity of cytotoxic cells generated by HIV vaccinia virus recombinants, susceptibility to CTL activity was compared among P815 target cells pulsed with peptides corresponding the V3 loop region of HIV isolates III_B, SF2, and MN. Only immunization with vP911 and vP921 induced HIV specific primary CTL activity (Table 5). Furthermore, HIV specific CTL activity was confined only to P815 target cells pulsed with peptide corresponding to the V3 loop of HIV isolate III_B. Similar results were obtained with in vitro stimulated, HIV specific secondary CTL activity induced by immunization with the vaccinia virus recombinants vP878, vP911, and vP921 (Table 6). Thus, HIV specific CTLs elicited by recombinant vaccinia viruses expressing various portions of the env gene of HIV isolate III_B recognize only target epitopes derived from the same antigenic isolate.

Lymphocyte Proliferation Responses to HIV Epitopes Following Immunization with Vaccinia Virus HIV Recombinants

Lymphocyte proliferation to antigens is an in vitro correlate of cell-mediated immunity. Presentation of the appro-

priate antigen induces cellular proliferation in the immune population of cells expressing receptors for the antigen. The initiation and continuation of proliferation requires the involvement of T helper lymphocytes via soluble mediators. To evaluate cell-mediated immunity to HIV antigens in mice immunized with recombinant vaccinia viruses expressing HIV antigens, spleen cells from mice immunized 27 days earlier were incubated for 5 days with peptides correlating to T helper lymphocyte epitopes designated T_1 and T_2, as well as with purified HIV gp160 (Table 7). No proliferative responses to the T helper cell epitopes T_1 and T_2 were observed in any of the spleen cell cultures. However, the spleen cells of mice previously immunized with vP921 vigorously responded to HIV gp160 as determined by the incorporation of [^{3}H]-thymidine. A stimulation index (SI) of greater than 2.0 is considered indicative of immunity. Thus, inoculation of mice with vP921 elicited cell-mediated immunity to HIV gp160.

Antibody Responses of Mice Inoculated with Vaccinia Virus HIV Recombinants

To evaluate humoral responses to HIV, mice were immunized at day 0 with one of the vaccinia virus HIV recombinants and received a secondary immunization at week 5. The mice were bled at various intervals through 9 weeks after the initial immunization. Pooled sera from each treatment group were assayed for antibodies to HIV by ELISA employing purified gp160 as antigen (Table 8). Primary antibody responses were generally modest, but detectable with the highest levels induced by vP911. Following the secondary immunization, the antibody titers of mice immunized with vP911 and vP921 increased and peaked at week 7 with titers of over 4,600 and 3,200, respectively, before declining slightly by week 9. Thus, two vaccinia virus HIV recombinants, vP911 and vP921, were capable of inducing a significant antibody response.

TABLE 1

Primary CTL activity of spleen cells from mice immunized with vaccinia virus recombinants against vaccinia virus infected targets and targets pulsed with peptide corresponding to the V3 loop region of HIV-1 gp120.

IMMUNIZATION		PERCENT CYTOTOXICITY TARGET P815	VAC	HIV V3
NONE		−3.5	−0.6	−4.8
	±	2.0	1.5	1.6
NYVAC		−4.4	9.5*	−5.9
	±	1.9	3.2	1.7
vP878		−4.9	7.1*	−4.0
	±	1.8	2.2	1.2
vP911		−4.0	4.6*	1.4
	±	2.5	2.0	5.1
vP921		−3.4	10.7*	15.5*
	±	0.9	1.5	2.8

E:T = 100:1
* P < 0.05 vs appropriate controls, Student's t-test

TABLE 2

Secondary CTL activity of spleen cells following in vitro stimulation with vaccinia virus recombinants.

IMMUNIZATION in vivo	in vitro	PERCENT CYTOTOXICITY TARGET P815	VAC	HIV V3
NONE	NONE	−0.1	1.9	0.5
	NYVAC	3.7	8.9	3.8
	vP878	4.6	9.0	5.5
	vP911	−1.7	2.9	4.8
	vP921	2.9	2.9	1.5
NYVAC	NONE	0.0	4.4	1.1
	NYVAC	3.5	47.8*	9.2
	vP878	6.3	44.1*	14.4
	vP911	7.9	48.6*	10.6
	vP921	6.8	50.8*	7.9
vP878	NONE	0.1	1.7	1.3
	NYVAC	10.2	58.5*	13.0
	vP878	11.6	57.9*	59.9*
	vP911	7.8	56.2*	40.8*
	vP921	4.9	42.0*	14.8
vP911	NONE	0.3	2.9	4.0
	NYVAC	6.2	50.7*	8.5
	vP878	5.9	50.9*	77.4*
	vP911	5.0	54.2*	82.6*
	vP921	10.9	55.0*	87.8*
vP921	NONE	2.9	5.0	9.4
	NYVAC	8.3	54.4*	22.7
	vP878	10.4	56.2*	85.6*
	vP911	8.7	58.2*	86.5*
	vP921	7.8	55.2*	81.0*

BALB/cJ spleen cells from mice immunized approximately 1 month earlier with the indicated vaccinia virus recombinants and were incubated with infected syngeneic spleen cells for 5 days and assayed for cytotoxicity at an effector to target cell ratio of 20.1.
* P < 0.05 compared to controls, Student's t-test.

TABLE 3

Anamnestic CTL responses of the spleen cells of mice administered a single inoculation of recombinant vaccinia or canarypox virus expressing the V3 loop of HIV gp120.

IMMUNIZATION PRIMARY in vivo	BOOSTER in vitro	PERCENT CYTOTOXICITY TARGET P815	Vac	CP	HIV V3
NONE	NONE	0.4	−2.5	−2.3	−1.5
	vP804	0.5	8.8	0.7	0.8
	vP878	1.8	6.1	0.4	1.6
	CP	5.8	4.2	4.9	0.4
	VCP95	4.4	2.6	6.1	0.1
	SB13S	−0.2	−0.7	−0.4	0.5
vP804	NONE	0.7	1.7	0.1	1.3
	vP804	5.5	43.5*	5.8	3.5
	vP878	3.6	42.5*	1.6	−0.3
	CP	8.5	7.0	5.6	3.9
	vCP95	5.8	5.3	4.4	4.0
	SB135	1.2	−0.9	−0.5	−0.2
vP878	NONE	0.2	−2.9	−0.8	−0.2
	vP804	5.3	56.4*	7.5	4.1
	vP878	6.7	60.2*	7.7	41.7*
	CP	8.7	13.4	9.4	4.7
	vCP95	7.1	10.5	8.7	19.0
	SB135	1.9	−0.7	−0.2	−1.4
cP	NONE	4.6	−0.6	2.3	−0.0
	vP804	11.0	17.7*	5.7	6.1
	vP878	7.1	14.6*	12.3	5.5
	CP	7.4	5.9	19.3*	3.1
	vCP95	6.8	5.4	20.4*	2.8

TABLE 3-continued

Anamnestic CTL responses of the spleen cells of mice administered a single inoculation of recombinant vaccinia or canarypox virus expressing the V3 loop of HIV gp120.

IMMUNIZATION		PERCENT CYTOTOXICITY			
PRIMARY	BOOSTER	TARGET			
in vivo	in vitro	P815	Vac	CP	HIV V3
	SB135	1.4	−0.4	0.8	−1.4
vCP95	NONE	−0.8	−2.2	−1.3	0.3
	vP804	9.4	26.4*	9.3	6.6
	vP878	10.4	22.5*	16.9	32.1*
	CP	8.8	7.2	20.0*	3.2
	VCP95	5.1	4.2	19.6*	7.8
	SB135	1.9	−1.5	−0.3	−1.2

Twenty-three days after immunization, the spleen cells were stimulated in vitro for 5 days with virus infected or peptide-pulsed syngeneic spleen cells and then assayed for specific cytotoxicity against virus infected or peptide-pulsed P815 target cells at an effector to target cell ratio of 20:1.
* P < 0.05 compared to appropriate controls, Student's t-test.

TABLE 4

Depletion of cytotoxic activity with monoclonal antibodies to CD8 plus complement.

IMMUNIZATION			PERCENT CYTOTOXICITY TARGETS		
in vivo	in vitro	TREATMENT	P815	VAC	HIV V3
NONE	NONE	NONE	1.1	1.5	−0.3
NONE	NYVAC	NONE	−7.4	0.4	−0.4
NONE	vP921	NONE	−0.2	1.1	−0.7
NYVAC	NONE	NONE	−3.1	−0.3	−1.4
NYVAC	NYVAC	NONE	−2.6	40.5	−0.3
NYVAC	vP921	NONE	3.3	31.4	−2.9
vP921	NONE	NONE	3.0	−1.3	−0.1
vP921	NYVAC	NONE	−4.9	25.9	12.2
vP921	vP921	NONE	−0.2	21.3	30.5
vP921	vP921	C'	4.6	20.1	22.9
vP921	vP921	anti-CD4	4.2	22.6	23.2
vP921	vP921	anti-CD8	−5.0	22.5	26.9
vP921	vP921	anti-CD4 + C'	10.0	26.6	30.1
vP921	vP921	anti-CD8 + C'	9.2	7.1	2.3

TABLE 5

Specificity of primary CTL activity for the V3 loop of HIV-1 isolate II_B following a single inoculation with HIV recombinant vaccinia viruses.

		PERCENT CYTOTOXICITY TARGET			
			V3 PEPTIDE		
IMMUNIZATION		P815	IIIB	SF2	MN
NONE		−2.7	−1.9	−0.9	−1.2
	±	−0.5	0.5	0.5	0.5
NYVAC		−1.6	−0.3	−0.6	−0.3
	±	−0.5	0.8	0.7	0.2
vP878		−2.8	0.5	−0.5	−1.2
	±	0.8	1.0	0.6	0.5
vP911		−2.6	7.5*	−0.5	−1.1
	±	0.2	3.2	0.5	0.4

TABLE 5-continued

Specificity of primary CTL activity for the V3 loop of HIV-1 isolate II_B following a single inoculation with HIV recombinant vaccinia viruses.

		PERCENT CYTOTOXICITY TARGET			
			V3 PEPTIDE		
IMMUNIZATION		P815	IIIB	SF2	MN
vP921		−2.5	12.5*	−0.1	−1.2
	±	0.7	3.6	0.5	0.5

Mice were administered a single iv inoculation with the indicated vaccinia virus recombinant and assayed for CTL activity 7 days later against P815 targets and P815 targets pulsed with one of three peptides corresponding to the V3 loop region of HIV-1 isolates III_B, SF2, and MN. Although assayed at effector to target cell ratios of 100:1, 50:1, and 25:1, only the 100:1 data are shown.
* P < 0.05 vs appropriate controls, Student's t-test

TABLE 6

Specificity of secondary CTL activity for the V loop of HIV-1 isolate III_B following a single inoculation with HIV recombinant vaccinia viruses.

IMMUNIZATION		PERCENT CYTOTOXICITY TARGET			
			V3 PEPTIDE		
in vivo	in vitro	P815	IIIB	SF2	MN
NONE	NONE	1.0	1.1	0.5	−0.0
	NYVAC	0.4	0.5	−0.6	−0.3
	vP878	0.2	0.2	−0.5	−1.0
	vP911	−1.5	0.3	−0.5	0.2
	vP921	−0.6	1.4	0.1	−0.5
NYVAC	NONE	−2.2	0.2	0.5	−1.0
	NYVAC	3.2	2.2	3.9	2.5
	vP878	4.4	5.9	5.0	6.1
	vP911	5.8	11.1	5.0	5.3
	vP921	5.0	6.5	2.9	2.9
vP878	NONE	0.1	−0.2	−0.9	−1.0
	NYVAC	3.0	4.8	4.4	4.5
	vP878	7.9	20.2	7.8	8.6
	vP911	4.8	7.8	4.5	4.7
	vP921	2.7	6.9	2.8	3.0
vP911	NONE	0.9	1.8	1.4	0.5
	NYVAC	8.8	8.3	8.1	6.6
	vP878	6.6	57.2	6.8	8.2
	vP911	4.6	63.7	2.9	4.2
	vP921	7.2	63.6	4.1	4.9
vP921	NONE	0.5	0.8	1.2	0.6
	NYVAC	4.4	7.9	7.5	6.0
	vP878	8.1	59.0	7.1	7.5
	vP911	6.4	71.4	7.9	6.6
	vP921	9.3	63.4	9.0	8.1

TABLE 7

Lymphocyte proliferative responses to HIV gp160 epitopes 27 days after a single immunization with HIV recombinant vaccinia viruses.

			COUNTS PER MINTUE HIV ANTIGEN							
			gp160 (μg)		T1 (μg)			T2 (μg)		
IMMUNIZATION		RPMI	1	10	1	5	10	1	5	10
NONE	MEAN	5,185	6,397	7,808	7,682	8,614	11,541	6,141	8,835	6,774
	± SD	1,020	2,174	2,596	1,274	2,033	2,036	2,103	1,883	2,806
	SI	1.0	1.2	1.5	1.0	1.1	1.5	1.0	1.4	1.1
NYVAC	MEAN	10,327	13,589	15,969	11,360	12,654	15,369	10,339	9,834	8,868
	± SD	1,543	3,323	4,583	1,352	2,272	1,821	762	1,731	502
	SI	1.0	1.3	1.5	1.0	1.1	1.4	1.0	1.0	0.9
vP878	MEAN	10,126	13,150	18,329	11,114	11,956	13,754	10,415	11,442	9,147
	± SD	1,269	1,103	4,245	1,217	1,106	1,568	335	1,288	1,033
	SI	1.0	1.3	1.8	1.0	1.1	1.2	1.0	1.1	0.9
vP911	MEAN	12,155	15,564	26,083	12,417	15,380	17,007	10,681	11,412	0,702
	± SD	1,307	9,707	16,327	873	1,847	6,266	2,428	3,201	1,468
	SI	1.0	1.3	2.1	1.0	1.2	1.4	1.0	1.1	1.0
vP921	MEAN	9,701	49,256 *	61,036 *	10,550	15,367	15,816	8,818	9,232	8,803
	± SD	2,601	23,673	25,866	3,447	3,481	7,176	954	2,265	2,860
	SI	1.0	5.1	6.3	1.0	1.5	1.5	1.0	1.0	1.0

SI - stimulation index.
* $P < 0.05$ compared to unstimulated control cultures Student's t-test

TABLE 8

HIV gp160 ELISA titers of mice immunized with HIV recombinant vaccinia viruses.

	WEEKS AFTER IMMMUNIZATION					
IMMUNIZATION	0	1	2	4	7	9
CONTROLS	22	32	32	38	36	33
NYVAC	38	36	37	28	50	45
vP878	20	43	27	46	65	63
vP911	0	0	90	453	4,614	3,263
vP921	0	26	25	77	2,614	1,689

EXAMPLE 3

EXPRESSION OF THE HIV-1 (ARV-2 OR SF-2 STRAIN) env GENE IN ALVAC, TROVAC AND NYVAC VECTORS

Plasmid Constructions

The lambda clone containing the entire HIV-1 (ARV-2 or SF-2 strain) genome was provided by J. Levy and was described previously (Sanchez-Pescador et al., 1985). The env sequences were subcloned into pUC13, creating plasmid pMP7MX373, which contains the sequences from -1 relative to the initiation codon (ATG) of the env gene product to 715 bp downstream of the termination codon (TAA) of the env gene. These env sequences were excised from pMP7MX373 by digestion with EcoRI and HindIII and inserted into the plasmid vector, pIBI25 (International Biotechnologies, Inc., New Haven, Conn.) generating plasmid pIBI25env.

Recombinant plasmid pIBI25env was used to transform competent *E. coli* CJ236 (dut- ung-) cells. Single-stranded DNA was isolated from phage derived by infection of the transformed *E. coli* CJ236 cells with the helper phage, MG408. This single-stranded template was used in in vitro mutagenesis reactions (Kunkel et al., 1985) with oligonucleotide MUENVT12 (SEQ ID NO:45) (5'-AGAGGGGAATTCTTCTACTGCAATACA-3'). Mutagenesis with this oligonucleotide generates a T to C transition and disrupts the T5CT motif at nucleotide positions 6929–6935 of the ARV-2 genome (Sanchez-Pescador et al., 1985). This mutation does not alter the amino acid sequence of the env gene and creates an EcoRI site, which was used to screen for mutagenized plasmid clones. Sequence confirmation was done by the dideoxynucleotide chain termination method (Sanger et al., 1977). The resultant mutagenized plasmid was designated as pIBI25mutenv11.

A 1.45 kb BglII fragment was derived from pIBI25mutenv11. This fragment contained the mutated env sequences. It was used to substitute for the corresponding unmutated fragment in pIBI25env. The resultant plasmid was designated as pIBI25mutenv8. Further modifications were made to pIBI25mutenv8. In vitro mutagenesis was performed to remove the sequence coding for the rex protein and the LTR sequence (LTR region) from the 3'-end of the gene and to delete the putative immuno-suppressive (IS) region amino acids 583 through 599 (SEQ ID NO:46:Leu-Gln-Ala-Arg-Val-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Arg-Asp-Gln-Gln-Leu) (Klasse et al., 1988). These reactions were done with the single-stranded template derived from pIBI25mutenv8 with oligonucleotides LTR2 (SEQ ID NO:47) (5'-TTGGAAAGGCTTTTGGCATGCCACGCGTC-3') and MUENSVISR (SEQ ID NO:48) (5'-ACAGTCTGGGGCATCAAGCAGCTAGGGATTTGGGGTTGCTCT-3'). Mutagenized clones were identified by hybridization and restriction analysis. A clone mutagenized such that it was deleted both of the IS and the LTR region and another deleted of the LTR was confirmed by nucleotide sequence analysis and designated pIBI25mut3env40 and pIBI25mut2env22, respectively.

A 3.4 kb SmaI/HindIII fragment containing the entire env gene was derived from pIBI25mut3env40 and from pIBI25mut2env22 and inserted into pCPCV1 and pFPCV2, digested with SmaI/HindIII. The plasmid pCPCV1 is an insertion plasmid which enables the generation of ALVAC recombinants with insertion occurring in the C3 locus. The plasmid, pFPCV2, is an insertion plasmid which enables the generation of TROVAC recombiants with insertion occurring in the F7 locus. Plasmids pCPCV1 and pFPCV2 have

been described previously in PCT International Publication No. WO 89/03429 published Apr. 20, 1989.

Oligonucleotide PROVECNS (SEQ ID NO:49) (5'-CCGTTAAGTTTGTATCGTAATGAAAGTGAAGGGGA-CCAGG-3') was used for in vitro mutagenesis reactions via the method of Mandecki (1986) to make a precise ATG:ATG construction with the VVH6 promoter and the env sequences. Potential mutants were screened for the loss of the SmaI site. Plasmid clones devoid of a SmaI site were identified and confirmed by nucleotide sequence analysis. Properly mutagenized plasmid clones were identified and designated as pCPenvIS+ or pCPenvIS– and pFPenvIS+ or pFPenvIS.

The HIV-1 env genes were excised from pCPenvIS– by digestion with NruI and HindIII. The two env fragments of 2.5 kb (envIS+) and 2.4 kb (envIS–), respectively, were isolated and blunt-ended by reaction with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. These fragments were ligated with the 3.5 kb fragment derived by digestion of pSIVenvVV with NruI and PstI with a subsequent blunting step with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. The plasmid pSIVenvVV contains the SIV env gene expression cassette regulated by the vaccinia virus H6 promoter in the ATI insertion locus. Digestion of pSIVenvVV with NruI and PstI excises the entire SIV env coding sequences and the 3'-most 20 bp of the promoter element. Ligation to the env IS– and env IS+ fragments restores the 20 bp of the H6 promoter and inserts the HIV-1 env gene into the ATI insertion plasmid. The resultant plasmids were designated as pAR5VV+ and pAR6VV– for env IS+ and env IS–, respectively.

In Vitro Recombination and Purification of Recombinants

Recombination was performed introducing plasmid DNA into infected cells by calcium phosphate precipitation both for ALVAC and for TROVAC recombinants, as previously described (Piccini et al., 1987). Plasmids pCPenvIS+ and pCPenvIS– were used to make recombinants vCP61 and vCP60, respectively. Plasmids pFPenvIS+ and pFPenvIS– for recombinants vFP63 and vFP62, respectively. The plasmids pAR5VV+ and pAR6VV– were used in in vitro recombination experiments with vP866 as rescue to yield vP939 and vP940, respectively. Recombinant plaques were selected by autoradiography after hybridization with a ^{32}P-labeled env specific probe and passaged serially three times to assure purity, as previously described (Piccini et al., 1987).

Expression of the HIV-1 env Gene

Six different recombinant viruses were prepared where the HIV env gene of the ARV-2 or SF-2 strain was inserted downstream from a vaccinia early-late promoter, H6. For simplicity, the two ALVAC-based recombinant viruses, vCP61 and vCP60, will be referred to as CPIS+ and CPIS–, the two TROVAC– based recombinants, vFP63 and vFP62, as FPIS+ and FPIS–, and the two NYVAC-based recombinants, vP939 and vP940, as VV– and VV+, respectively.

All the constructs were precise, in that, the ATG initiation codon of the HIV-1 env gene was superimposed on the ATG of the vaccinia H6 promoter. Moreover, all extraneous genetic information 3' to the termination codon was eliminated. CPIS–, FPIS–, and VV– were all obtained by deletion of a 51 bp region, corresponding to amino acids 583–599, located near the 5' portion of the gp41 gene product. This region shares homology with putative immunosuppressive regions (Klasse et al., 1988; Ruegg et al., 1989a,b) occurring in the transmembrane polypeptide of other retrovirus glycoproteins (Cianciolo et al., 1985; Ruegg et al., 1989a,b).

Expression analyses with all six recombinant viruses were performed in CEF, Vero, and MRC-5 cell monolayers, radioimmunoprecipitation experiments using pooled sera from HIV seropositive individuals were performed as described above. All six recombinants directed the synthesis of the HIV-1 gp160 envelope precursor. The efficiency of processing of gp160 to gp120 and gp41, however, varied between cell types and was also affected by deletion of the immunosuppressive region. Recognition of gp41 by the pooled sera from HIV seropositive individuals also varied between the virus background and the cell type.

EXAMPLE 4

EXPRESSION OF THE HIV-2 (ISSY STRAIN) env GENE IN NYVAC

Expression of gp160

Oligonucleotides HIV25PA (SEQ ID NO:50) (5'-ATGAGTGGTAAAATTCAGCTGCTTGTTGCCTTTCT-GCTAACTAGTGCTTGCTTA-3') and HIV25PB (SEQ ID NO:51) (5'-TAAGCAAGCACTAGTTAGCAGAAAGGC-AACAAGCAGCTGAATTTTACCACTCAT-3') were annealed to constitute the initial 54 bp of the HIV-2 ISSY strain (Franchini et al., 1989) env coding sequence. This fragment was fused 3' to a 129 bp fragment derived by PCR with oligonucleotides H65PH (SEQ ID NO:52) (5'-ATCATCAAGCTTGATTCTTTATTCTATAC-3') and H63PHIV2 (SEQ ID NO:53) (5'-CAGCTGAATTTTACCACTCATTACGATACAAACTT-AACG-3') using pTP15 (Guo et al., 1989) as template. The fusion of these two fragments was done by PCR using oligonucleotides HIV25PC (SEQ ID NO:54) (5'-TAAGCAAGCACTAGTTAG-3') and H65PH (SEQ ID NO:52). The 174 bp PCR derived fragment was digested with HindIII and SacI and inserted into pBS-Sk (Stratagene, La Jolla, Calif.) digested with HindIII and SacI. The resultant fragment was designated pBSH6HIV2. The insert was confirmed by nucleotide sequence analysis.

The 3' portion of the HIV-2 env gene was also derived by PCR. In this reaction a 270 bp fragment was amplified with oligonucleotides HIV2B1 (SEQ ID NO:55) (5'-CCGCCTCTTGACCAGAC-3') and HIV2B2 (SEQ ID NO:56) (5'-ATCATCTCTAGAATAAAAATTACAGGA-GGGCAATTTCTG-3') using pISSY-KPN (provided by Dr. Genoveffa Franchini, NCI-NIH, Bethesda, Md.) as template. This fragment fragment was digested with BamHI and XbaI. The 150 bp fragment derived from this digestion contained a 5' BamHI and a 3' XbaI cohesive end. The fragment was engineered to contain a T5NT sequence motif known to be recognized as vaccinia virus early transcription termination signal (Yuen et al., 1987), following the termination codon (TAA).

The majority of the HIV-2 env gene was obtained from pISSY-KPN by digestion with SacI and BamI. The 2.7 kb fragment generated by this digestion was coinserted into pBS-SK digested with SacI and XbaI with the 150 bp BamHI/XbaI fragment corresponding to the 3' end of the gene. The resultant plasmid was designated pBSHIV2ENV.

The 174 bp SpeI/HindIII fragment from pBSH6HIV2 and the 2.5 kb SpeI/XbaI fragment from pBSHIV2ENV were ligated into pBS-SK digested with HindIII and XbaI to yield pBSH6HIV2ENV. The 2.7 kb HindIII/XbaI insert from pBSH6HIV2ENV was isolated and blunt-ended with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTP. The blunt-ended fragment was inserted into a SmaI digested pSD541VC insertion vector. The resultant plasmid was designated as pATIHIV2ENV. This plasmid was used in vitro recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP920.

Immunoprecipitation analysis was performed to determine whether vP920 expresses authentic HIV-2 gp160.

Lysates from the infected cells were analyzed for HIV-2 env gene expression using pooled serum from HIV-2 seropositive individuals (obtained from Dr. Genoveffa Franchini, NCI-NIH, Bethesda, Md.). The sera was preadsorbed with vP866 infected Vero cells. The preadsorbed human sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4° C. with the human sera from seropositive individuals bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris(pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV-2 seropositive individuals specifically precipitated the HIV-2 gp160 envelope glycoprotein from vP920 infected cells. Furthermore, the authenticity of the expressed HIV-2 env gene product was confirmed, since the gp160 polyprotein is processed to the mature gp120 and gp41 protein species. No HIV-specific protein species were precipitated from mock-infected cells or cells infected with the parental virus, vP866. Also, supportive of the proper expression of the HIV-2 env by vP920 was the observation by an immunofluorescence assay that the gene product is expressed on the surface of vP920 infected cells.

Expression of gp120

The plasmid pBSH6HIV2 containing the vaccinia virus H6 promoter fused to the 5'-end of the HIV-2 env gene was digested with SpeI and HindIII to liberate the 180 bp fragment containing these sequences. This fragment was ligated into pBS-SK digested with HindIII and XbaI along with the 1.4 kb SpeI/XbaI fragment of pBSHIV2120A to yield pBSHIV2120B.

The plasmid pBSHIV2120A was derived by initially deriving the 3' portion of the gp120 coding sequence by PCR. The PCR was performed using oligonucleotides HIV2120A (SEQ ID NO:57) (5'-ATCATCTCTAGAATAAAAATTATCTCTTATGTCT-CCCTGG-3') and HIV2120B (SEQ ID NO:58) (5'-AATTAACTTTACAGCACC-3') with pISSY-KPN as template. The PCR-derived fragment was digested with EcoRI and XbaI to yield a 300 bp fragment which contained a 5'-EcoRI cohesive end and a 3'-XbaI cohesive end. The fragment was engineered with a translation termination sequence (TAA) and a T5NT sequence motif just 5' to the XbaI site. The 300 bp XbaI/EcoRI PCR fragment was ligated into pBS-SK digested with SacI/XbaI along with a 1.4 kb SacI/EcoRI fragment derived from pISSY-KPN to generate pBSHIV2120A.

The plasmid pBSHIV2120B was digested with HindIII and XbaI to generate a 1.8 kb fragment containing the HIV-2 gp120 coding sequence juxtaposed 3' to the vaccinia virus H6 promoter. This fragment was blunted with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. The blunt-ended fragment was ligated to SmaI digested pSD541VC to generate pATIHIV2120. This plasmid was used in in vitro recombination experiments to yield vP922.

Immunoprecipitation experiments with vP922 infected cells were performed as described above for the expression of the entire HIV-2 env gene. No HIV-specific species were precipitated from mock infected or vP866 infected Vero cells. A protein species of 120 kDa was, however, precipitated from lysates derived from cells infected with vP922.

EXAMPLE 5

EXPRESSION OF SIV GENES IN NYVAC

Generation of NYVAC/SIV gp140 Recombinant

A plasmid pSS11E containing the SIV ($_{mac142}$) env gene was obtained from Dr. Genoveffa Franchini (NCI-NIH, Bethesda, Md.). This plasmid was digested with HindIII and PstI to liberate a 2.2 kbp fragment containing from nucleotide 220 of the SIV env gene to a region 160 bp downstream from the translation termination codon. It should be noted that an expression cassette containing this fragment will result in the expression of a gp140 protein species rather that a gp160 species. This 40% deletion of the transmembrane region results from a premature termination at nucleotide 7,934 of the genome (Franchini et al., 1987). Such premature terminations of the env gene product are noted after propagation of SIV in culture (Kodama et al., 1989).

The amino portion of the gene was derived by PCR using pSS11E as template and oligonucleotides SIVENV1 (SEQ ID NO:59) (5'-CGATATCCGTTAAGTTTGTATCGTAA-TGGGATGTCTTGGGAATC-3') and SIVENV2 (SEQ ID NO:60) (5'-CAAGGCTTTATTGAGGTCTC-3'). The resultant 250 bp fragment contains the 5'-most 230 bp of the SIV env gene juxtaposed downstream from the 3'-most 20 bp of the vaccinia virus H6 promoter (3'-end of NruI site). A 170 bp fragment was obtained by digestion of the fragment with HindIII, which removes 80 bp of SIV env sequences.

The sequences containing the remainder of the SIV env gene following the premature termination signal were derived by PCR from pSS35E (obtained from Dr. Genoveffa Franchini). This plasmid contains sequences containing the C-terminal portion of the SIV env gene into the LTR region downstream from the env gene. The oligonucleotides used to derive the 360 bp fragment were SIVENV3 (SEQ ID NO:61) (5'-CCTGGCCTTGGCAGATAG-3') and SIVENV4A (SEQ ID NO:62) (5'-ATCATCGAATTCAAAAATATTACAAAGAGCGTGA-GCTCAAGTCCTTGCCTAATCCTCC-3'). This fragment was digested with PstI and EcoRI to generate a 260 bp fragment having a 5' PstI cohesive end and a 3'- EcoRI cohesive end.

The 2.2 kb HindIII/PstI fragment from pSS11E, the 170 bp NruI/HindIII fragment containing the 5' end of the gene, and the 260 bp PstI/EcoRI containing the 3' end of the gene were ligated with a 3.1 kb NruI/EcoRI fragment derived from pRW838. pRW838 contains the vaccinia virus H6 promoter linked to the rabies G gene flanked by canarypox-virus sequences which enable the insertion of genes into the C5 locus. Digestion with NruI and EcoRI liberates the rabies G gene and removes the 3'-most 20 bp of the H6 promoter. The resultant C5 insertion plasmid containing the SIV env gene linked to the vaccinia H6 promoter was designated as pC5SIVENV.

The plasmid, pC5SIVENV, was digested with HindIII and EcoRI to liberate a 2.2 kb fragment, containing from nucleotide 150 of the SIV env gene to the end of the entire gene. PCR was used to derive the vaccinia H6 promoter/SIV env linkage from pC5SIVENV with oligonucleotides MPSYN286 (SEQ ID NO:63) (5'-CCCCCCAAGCTTTTTATTCTATACTT-3') and SIVENV2 (SEQ ID NO:64) (5'-CAAGGCTTTATTGAGGTCTC-3'). The 320 bp fragment was digested with HindIII to derive a 240 bp fragment. The

2.2 kb HindIII/EcoRI and the 240 bp HindIII fragment were coligated into pC3I digested with HindIII and EcoRI. The resultant plasmid containing the HindIII fragment in the proper orientation relative to the SIV env coding sequence was designated pC3SIVEM. The plasmid pC3I was derived as follows. The nucleotide sequence analysis of an 2.5 kb BglII canarypoxvirus genomic fragment revealed the entire C3 open reading frame and the 5' and 3' flanking regions. In order to construct a donor plasmid for insertion of foreign genes into the C3 locus with the complete excision of the C3 open reading frame, PCR primers were used to amplify the 5' and 3' sequences relative to C3. Primers for the 5' sequences were RG277 (SEQ ID NO:65) (5'-CAGTTGGTACCACTGGTATTTTATTTCAG-3') and RG278 (SEQ ID NO:66) (5'-TATCTGAATTCCTGCAGCCCGGGTTTTTATAGCTAATTAGTCAAATGTGAGTTAATATTAG-3').

Primers for the 3' sequences were RG279 (SEQ ID NO:67) (5'-TCGCTGAATTCGATATCAAGCTTATCGATTTTTATGACTAGTTAATCAAATAAAAAGCATACAAGC-3') and RG280 (SEQ ID NO:68) (5'-TTATCGAGCTCTGTAACATCAGTATCTAAC-3'). The primers were designed to include a multiple cloning site flanked by vaccinia transcriptional and translational termination signals. Also included at the 5'-end and 3'-end of the left arm and right arm were appropriate restriction sites (Asp718 and EcoRI for left arm and EcoRI and SacI for right arm) which enabled the two arms to ligate into Asp718/SacI digested pBS-SK plasmid vector. The resultant plasmid was designated as pC3I.

The plasmid pC3SIVEM was linearized by digestion with EcoRI. Subsequent partial digestion with HindIII liberated a 2.7 kb HindIII/EcoRI fragment. This fragment was blunt-ended by treatment with Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs. The fragment was ligated into pSD550VC digested with SmaI. The resultant plasmid was designated as pSIVEMVC. This plasmid was used in in vitro recombination experiments with vP866 as rescue virus to generate vP873. vP873 contains the SIV env gene in the I4L locus.

Generation of a NYVAC/gag/pol and gag Recombinants

A plasmid, pSIVAGSS11G, containing the SIV cDNA sequence encompassing the gag and pol genes was obtained from Dr. Genoveffa Franchini (NCI-NIH, Bethesda, Md.). The gag and pol genes from this plasmid were juxtaposed 3' to the vaccinia I3L promoter between vaccinia tk flanking arms. This was accomplished by cloning the 4,800 bp CfoI/TaqI fragment of pSIVGAGSS11G, containing the gag and the oligonucleotides SIVL1 (SEQ ID NO:69) (5'-TCGAGTGAGATAAAGTGAAAATATATATCATTATATTACAAGTACAATTATTTAGGTTTAATCATGGGCG-3') and SIVL2 (SEQ ID NO:70) (5'-CCCATGATTAAACCTAAATAATTGTACTTTGTAATATAATGCTATATATTTTCACTTTATCT- CAC -3') corresponding to the I3L promoter into the 4,070 bp XhoI/AccI fragment of pSD542, a derivative of pSD460 (FIG. 1). The plasmid generated by this manipulation was designated pSIVG1.

To eliminate the pol gene, a 215 bp PCR fragment was derived from pSIVGAGSS11G using oligonucleotides SIVP5 (SEQ ID NO:71) (5'-AATCAGAGAGCAGGCT-3') and SIVP6 (SEQ ID NO:72) (5'-TTGGATCCCTATGCCACCTCTCT-3'). The PCR-derived fragment was digested with BamHI and StuI and ligated with the 5,370 bp partial BamHI/StuI fragment of SIVG1. This resulted in the generation of pSIVG2. pSIVG2 was used in in vitro recombination experiments with vP873 as rescue virus to yield vP948.

The plasmid to insert both gag and pol into NYVAC-based vectors was engineered in the following manner. pSIVG1, described above, contains extraneous 3'-noncoding sequences which were eliminated using a 1 kb PCR fragment. This fragment was generated from plasmid pSIVGAGSS11G with the oligonucleotides SIVP5 and SIVP6. This PCR derived fragment containing the 3' end of the pol gene was digested with BamHI and HpaI. The 1 kb BamHI/HpaI fragment was ligated to the 7,400 bp partial BamHI/HpaI fragment of pSIVG1 to yield pSIVG4.

Sequence analysis of pSIVG4 revealed a single base pair deletion within the pol gene. To correct this error the 2,300 bp BglII/StuI fragment from pSIVG1 was inserted into the 6,100 bp partial BglII/StuI fragment of pSIVG4 to yield pSIVG5. The plasmid, pSIVG5, was used in in vitro recombination experiments with vP873 as rescue to generate vP943.

Generation of NYVAC/SIV p16 and p28 Recombinants

The pol gene and the portion of the gag gene encoding p28, p2, p8, p1, and p6 were eliminated from pSIVG1. This was accomplished by cloning the oligonucleotides SIVL10 (SEQ ID NO:73) (5'-AGACCAACAGCACCATCTAGCGGCAGAGGAGGAAATTACTAATTTTTATTCTAGAG-3') and SIVL11 (SEQ ID NO:74) (5'-GATCCTCTAGAATAAAAATTAGTAATTTCCTCCTCTGCCGCTAGATGGTGCTGTTGGT-3') into the 4,430 bp AccI/BamHI fragment of pSIVG1 to generate pSIVG3. This plasmid contains an expression cassette for the SIV p16 gene product expressed by the vaccinia I3L promoter.

The entomopoxvirus 42 kDa-promoted SIV p28 gene (5' end only) was inserted downstream from the I3L-promoted p16 gene. This was accomplished by cloning the 360 bp BspMI/BamHI fragment of pSIVG1, containing the 5' end of the p28 gene, the oligonucleotides pSIVL14 (SEQ ID NO:75) (5'-TAGACAAAATT GAAAATATATAATTACAATATAAAATGCCAGTACAACAAATAGGTGGTAACTATGTCCACCTGCC ATT-3') and SIVL15 (SEQ ID NO:76) (5'-GCTTAATGGCAGGTGGACATAGTTACCACCTATTTGTTGTACTGGCATTTTATATTGTAATTATATATTTTCAATTTTGT-3'), containing the entomopox 42 kDa promoter into the 4,470 bp partial XbaI/BamHI fragment of pSIVG3. The resultant plasmid was designated as pSIVG6.

The 3' portion of the p28 gene was then inserted into pSIVG6. A 290 bp PCR fragment, containing the 3' end of the SIV p28 gene, was derived from pSIVGI using oligonucleotides SIVP12 (SEQ ID NO:77) (5'-TGGATGTACAGACAAC-3') and SIVP13 (SEQ ID NO:78) (5'-AAGGATCCGAATTCTTACATTAATCTAGCCTTC-3'). This fragment was digested with BamHI and ligated to the 4,830 bp BamHI fragment of pSIVG6. The resultant plasmid, pSIVG7, was used in in vitro recombination experiments with vP866 and vP873 as rescue experiments to generate vP942 and vP952, respectively.

Expression Analyses

The SIV gp140 env gene product is a typical glycoprotein associated with the plasma membrane of infected cells. It is expressed as a polyprotein of 140 kDa that is proteolytically cleaved to an extracellular species of 112 kDa and a transmembrane region of 28 kDa (Franchini et al., 1987). Immunofluorescence analysis using sera from rhesus macaques seropositive for SIV followed by fluorescein conjugated

rabbit anti-monkey IgG demonstrated expression of the env gene product on the surface of recombinant infected Vero cells. Surface expression was not detectable on the surface of mock infected cells or cells infected with the NYVAC (vP866) parent virus. Furthermore, cells infected with recombinants containing only gag genes were not shown to express any SIV components on the surface. Surface expression in cells infected with vP873, vP943, vP948 and vP952 all demonstrated surface expression and significantly, all contain the SIV env gene.

The authenticity of the expressed SIV gene products (env and gag) in Vero cells infected with the NYVAC/HIV recombinants was analyzed by immunoprecipitation as described above, except all samples were harvested at 17 hours post infection by the addition of 1 ml of 3×Buffer A. Lysates from the infected cells were analyzed with pooled sera from SIV seropositive rhesus macaques or a monoclonal antibody specific for gag p24 gene product (both obtained from Dr. Genoveffa Franchini, NCI-NIH, Bethesda Md.).

Immunoprecipitation with the SIV seropositive macaques sera was performed in the following manner. The macaque sera was incubated with a protein A-sepharose at 4° C. for 16 hours. After washing with buffer A, the sera bound to protein A sepharose was added to lysates precleared with normal monkey sera and protein A sepharose. Following an overnight incubation at 4° C. the precipitates were washed 4×with buffer A and 2×with LiCl/urea buffer. To dissociate the precipitated protein from the antibody, the samples were boiled in 80 μl 2×Laemmli buffer for 5 minutes. The samples were fractionated on a 12.5% gel using the Dreyfuss gel system (Dreyfuss et al., 1984). The gel was fixed and treated with 1M Na-salicylate for fluorography. All the recombinants containing SIV genes were expressing the pertinent gene products. The NYVAC recombinants vP873, vP943, vP948 and vP952 which contain the SIV env gene all expressed the authentic gp140. However, it is difficult to assess the processing of the gp140 protein to the 112 kDa and 28 kDa mature forms. No species with an apparent molecular weight of 140 kDa was precipitated by macaque anti-SIV sera from mock infected Vero cells, vP866 infected Vero cells and Vero cells infected with NYVAC/SIV recombinants not containing the SIV env gene. Expression of the SIV gag encoded gene products by vP942, vP943, vP948, and vP952 was demonstrated using the pooled sera from macaques infected with SIV and the monoclonal antibody specific to the p28 gag component. Expression of the entire p55 gag protein without the pol region, which contains the protease function, by NYVAC (vP948) in Vero cells is evident. These results demonstrate that lack of SIV protease expression prevents the complete proteolysis of p55 into its mature form. This is demonstrated much more clearly when a monoclonal antibody specific to p28 was used to precipitate gag specific gene products from vP948 infected Vero cells. Contrary to this result, expression of SIV gag with the pol gene (includes protease in vP943 infected Vero cells) enabled the expressed p55 gag precursor polypeptide to be proteolytically cleaved to its mature forms.

Expression of both the p16 and p28 SIV gene products in vP942 and vP952 infected Vero cells was demonstrated using the pooled sera from macaques infected with SIV. Using the monoclonal antibody specific to p24 obviously only recognized the p28 expressed component.

Certain Materials and Methods Which Relate to Examples 6 to 8.

Assay for Cytotoxic T Lymphocytes and In Vitro Stimulation of Memory Precursors of Cytotoxic T Lymphocytes. Six week old female BALB/c mice were inoculated intravenously with 5×10^7 pfu of vaccinia virus (NYVAC), recombinant vaccinia virus expressing HIV-1 (IIIB) env (vP911), canarypoxvirus (ALVAC), or with recombinant canarypoxvirus expressing HIV-1 (IIIB) env (vCP112). Seven days later, the spleen cells were assayed for primary CTL activity against unmodified P815 cells or P815 cells that had been incubated overnight with a peptide corresponding to the hypervariable V3 loop region of HIV-1 (IIIB) gp120. Twenty-two days after the initial immunization, the spleen cells of the experimental mice were incubated with poxvirus infected stimulator spleen cells and assayed for memory CTL activity against peptide pulsed targets as before. To determine secondary CTL activity, 29 days after the primary immunization mice received a second inoculation of identical dosage and content as the first. Five days later, the spleen cells were assayed for cytolytic activity against peptide pulsed targets. For cytotoxicity assays, H-2^d P815 murine mastocytoma cells were incubated overnight in medium (Minimum Essential Medium containing Earle's salts and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with or without 20 μg/ml V3 peptide (CNTRKRIRIQRGPGRAFVTGK, American Bio-Technologies, Inc.) (SEQ ID NO:79). The following morning, the P815 cells were washed by centrifugation and labeled for 1 hr at 37° C. in 100 μCi of $Na_2{}^{51}CrO_4$ per 2×10^6 cells. Intact spleens were aseptically removed from euthanized mice, bathed in ice cold Hank's Balanced Salt Solution, and disrupted into single cell suspensions using a Stomacher blender. The spleen cell suspensions were washed several times by low speed centrifugation and resuspended in Assay Medium (RPMI 1640 containing 10% fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, 5×10^{-5}M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). For memory CTL activity, the spleen cells from immunized mice were resuspended in Stimulation Medium (Minimum Essential Medium with Earle's salts containing 10% fetal bovine serum, 2 mM L-glutamine, 10^{-4}M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) and stimulated in vitro in upright 25 cm^2 tissue culture flasks with naive syngeneic stimulator spleen cells that had been infected with one of the poxviruses or poxvirus recombinants. After five days at 37° C., the cells were washed, counted, and resuspended in Assay Medium. ^{51}Chromium labelled target cells were added to titrated effector cells in 96-well microtiter plates for a 4 hr ^{51}Cr release assay. Effector to target cell ratios (E:T) shown for the three assays were 100:1 (primary), 20:1 (memory), and 50:1 (secondary). Percent cytotoxicity was calculated as described above, i.e., (experimental ^{51}Cr release−spontaneous ^{51}Cr release)/(maximum ^{51}Cr release−spontaneous ^{51}Cr release)×100. Maximum release was determined by the addition of 5% sodium dodecyl sulfate to target cells while spontaneous release was determined by incubating target cells in the absence of effector cells. In none of the experiments presented did spontaneous release of ^{51}Cr from target cells exceed 20% of maximum ^{51}Cr release. Error bars in FIG. 7 represent 1 standard deviation from the mean. $P<0.05$, Student's t-test compared to appropriate vaccinia or canarypoxvirus immunized mice.

Cell Surface Phenotype of Cytotoxic Effector Cells. Mice spleen cells were immunized with vaccinia virus or canarypox virus vectors (NYVAC, ALVAC) or with vaccinia virus or canarypox virus recombinants expressing HIV IIIB env (vP911, vCP112). A second inoculation was administered 30 days after the first. Prior to addition to V3 peptide pulsed

targets, the spleen cells were treated with monoclonal antibodies or alloantiserum to murine T-lymphocyte surface antigens in a two-stage protocol. Briefly, the spleen cells were resuspended at 10^7 viable cells per ml of Cytotoxicity Medium (RPMI 1640 containing 0.2% BSA and 5 mM HEPES) to which was added alloanti-Thy 1.2 (Cedarlane), monoclonal anti-CD4 (172.4, K. J. Weinhold, Duke University Medical Center), or monoclonal anti-Lyt 2.2 (Cedarlane). After 30 min at 5° C., the cells were washed and resuspended in the original volume of Cytotoxicity Medium, divided into two equal portions with or without complement (Rabbit Lo-Tox M, Cedarlane) and incubated at 37° C. for 45 min. The cells were then washed in Assay Medium and, based on the pre-treatment cell densities, resuspended in volumes of Assay Medium approximating effector to target cell ratios of 100:1 (primary), 10:1 (memory), or 80:1 (secondary) before addition to a 5 hr ^{51}Cr release assay. Error bars in FIG. 10 represent 1 standard deviation from the means.

Specificity of CTL Antigen Receptor Recognition of the V3 Loop Region of HIV IIIB gp120. Cytotoxic T lymphocytes and memory precursors of cytotoxic T lymphocytes were generated by inoculation of mice with vCP112 as described above. Assays for cytotoxic T lymphocytes were performed as described above except that P815 target cells were pulsed overnight with V3 peptide from HIV-1 IIIB (CN TRKRIRIQRGPGRAFVTGK) (SEQ ID NO:79), MN (CN KRKRIHIGPGRAFYTTKN) (SEQ ID NO:80), or SF2 (CN TRKSIYIGPGRAFHTTGR) (SEQ ID NO:81). Effector to target cell ratios were 100:1 (primary), 20:1 (memory), and 50:1 (secondary).

Antibody Responses to HIV-1 (IIIB) gp120. The wells of ELISA plates (Immulon II) were coated overnight at 4° C. with 0.5 μg of partially purified HIV-1 (IIIB) gp120 (Dr. G. Franchini, NCI-NIH, Bethesda, Md.) in carbonate buffer, pH 9.6. The plates were then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST). The plates were then blocked for 2 hr at 37° C. with PBST containing 1% bovine serum albumin (BSA). After washing with PBST, sera were initially diluted 1:20 with PBST containing 0.1% BSA (dilution buffer). The sera were further 2-fold serially diluted in the wells of the ELISA plate. The plates were incubated at 37° C. for 2 hr and washed with PBST. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins (DAKO) was diluted 1:2000 in dilution buffer and added to the wells of the ELISA plated and incubated at 37° C. for 1 hour. After washing with PBST, OPD (o-phenylenediamine dihydrochloride) in substrate buffer was added and the color was allowed to develop at ambient temperature for about 20 min. The reaction was extinguished by the addition of 2.5M H_2SO_4. The absorbance at 490 nm was determined on a Bio-Tek EL-309 ELISA reader. The serum endpoint was defined as the reciprocal of the dilution giving an absorbance value of 0.4.

EXAMPLE 6

A RECOMBINANT CANARYPOXVIRUS EXPRESSING HIV env ELICITS HIV-SPECIFIC CYTOTOXIC T LYMPHOCYTE ACTIVITY

Seven days after the initial inoculation with the HIV canarypoxvirus recombinant (vCP112; defined in Example 2), cytotoxic responses of spleen cells against HIV V3 peptide pulsed target cells were roughly equivalent to the cytotoxic responses elicited by the same dose, 5×10^7 pfu, of the NYVAC recombinant, vP911 (Example 2) expressing the same HIV env gene (FIG. 7). Following appropriate in vitro stimulation or a second inoculation, the levels of cytotoxicity of the spleen cells of mice given the canarypoxvirus recombinant increased and were comparable to spleen cells from mice similarly administered the NYVAC recombinant. No such cytotoxic responses were detected from spleen cells of mice inoculated with the non-recombinant NYVAC or ALVAC vectors confirming the requirement for immunization with a poxvirus recombinant expressing the HIV env gene. Furthermore, no cytotoxic reactivity was detected against unmodified P815 cells from the spleen cells of any of the mice regardless of the inoculation regimen. Thus, only mice inoculated with recombinant NYVAC or, more significantly, recombinant ALVAC expressing the env coding sequence from HIV-1 demonstrated V3-specific cytotoxic responses.

EXAMPLE 7

CHARACTERIZATION OF CYTOTOXIC EFFECTOR CELLS

To determine the identity of the spleen cells associated with the lysis of HIV-1 V3 peptide pulsed target cells, mice were immunized with vCP112. After each immunization, or in vitro stimulation 21 days after the first inoculation a two-step depletion procedure was performed, and the spleen cells were assessed for cytotoxicity against V3 peptide pulsed P815 cells. Mice inoculated with the canarypox vector ALVAC did not generate spleen cells capable of killing peptide pulsed targets. Following a single immunization, vCP112 induced spleen cells able to kill V3 peptide pulsed targets. The lytic effector cells were sensitive to treatment with anti-murine Thy 1.2 or Lyt 2.2 plus complement and were resistant to anti-CD4. FIG. 10 shows the sensitivity of the cytotoxic effector cells from spleen cells of mice immunized with vCP112 to antibodies against cytotoxic T lymphocyte cell surface antigens Thy 1.2 and Lyt 2.2. Neither complement nor any of the monoclonal antibodies or alloantisera alone affected the cytolytic action of these cells. Similar results were obtained five days after a second immunization administered on day 30. Twenty-one days after a single inoculation, in vitro stimulation with vCP112 infected syngeneic spleen cells gave rise to lytic effector cells only partially sensitive to anti-Thy 1.2 although completely sensitive to anti-Lyt 2.2 and resistant to anti-CD4. These Thy 1.2-, CD4-, Lyt 2.2+effector cells are not seen following in vitro stimulation with vP911 of spleen cells from vCP112 inoculated mice. Nonetheless, it is clear that HIV V3 loop specific cytotoxicity was mediated by a population of T lymphocytes expressing Thy 1.2 and Lyt 2.2, but not CD4. This cell surface phenotype is characteristic of classical cytotoxic T lymphocytes.

EXAMPLE 8

SPECIFICITY OF CTL ANTIGEN RECEPTOR RECOGNITION OF THE V3 LOOP REGION OF HIV gp120

To examine the specificity of cytotoxic cells generated by the HIV canarypox virus (ALVAC) recombinant, vCP112 susceptibility to CTL activity was compared among P815 target cells pulsed with peptides corresponding the V3 loop region of gp120 of HIV isolates IIIB, MN, or SF2. HIV specific primary CTL activity was confined only to P815 target cells pulsed with peptide corresponding to the V3 loop of HIV isolate IIIB, but not target cells pulsed with peptides corresponding to the V3 loop region of gp120of HIV isolates MN or SF2, as shown in FIG. 8 which illustrates the

specificity of cytotoxic T lymphocyte antigen receptor for the HIV IIIB hypervariable V3 loop of gp120, but not for the V3 loop of HIV MN or SF2. Similar results were obtained with in vitro stimulated, HIV specific memory CTL activity and secondary CTL activity induced by immunization with the ALVAC recombinant vCP112. Thus, HIV specific CTLs elicited by a recombinant canarypox virus expressing the env gene of HIV isolate IIIB recognize only target epitopes derived from the same antigenic isolate. These results clearly indicate the exquisite specificity of the lymphocyte effector cells generated by immunization with the HIV canarypox virus recombinant and eliminate such nonspecific effector mechanisms as natural killer (NK) cell activity.

EXAMPLE 9

ANTIBODY RESPONSES OF MICE INOCULATED WITH NYVAC- and ALVAC-BASED HIV RECOMBINANTS

To evaluate humoral responses to HIV, mice were immunized at day 0 with a NYVAC HIV recombinant or canarypoxvirus (ALVAC) recombinant and received a secondary immunization at week four. The mice were bled at various intervals through 20 weeks after the initial immunization. Pooled sera from each treatment group were assayed for antibodies to HIV by ELISA employing purified gp120 as antigen; the results are shown in FIG. 9 which provides the antibody responses to HIV IIIB gp120 of mice immunized with vectors (NYVAC, ALVAC) or with NYVAC recombinant vP911 or ALVAC recombinant (vCP112) expressing HIV-1 env, wherein the inverted triangle indicates the time of administration of the second inoculation. Primary antibody responses were generally modest, but detectable. Following the secondary immunization, the antibody titers of mice immunized with both vP911 and vCP112 increased and peaked at week six with titers of over 10,000. These antibody titers remained at approximately the same levels throughout the duration of the study. Thus, an ALVAC HIV recombinant, vCP112, was capable of inducing a significant antibody response.

Inoculation of mice with ALVAC expressing the env gene of HIV-1 elicits spleen cell reactivity with characteristics of cytotoxic T lymphocytes: the requirement for immunization, cell surface phenotype, memory, and elegant epitope specificity. Furthermore, antibody responses to HIV-1 gp120 are induced by inoculation with this ALVAC recombinant.

EXAMPLE 10

DERIVATION OF NYVAC- AND ALVAC-BASED HIV-1 RECOMBINANTS AND EXPRESSION OF HIV-1(MN) env BY ALVAC AND NYVAC

HIV-1(MN) env sequences were derived from plasmid pMN1.8–9 and pMN1.8–10 which contain a 1774 bp and 1803 bp subfragment from a genomic cDNA clone of HIV-1(MN), respectively. These plasmids were provided by the laboratory of Dr. R. C. Gallo (NCI-NIH). A 1,026 bp KpnI/EcoRI fragment was derived by amplifying these sequences from pMN1.8–9 by PCR using oligonucleotides HIVMN6 (SEQ ID NO:82) (5'-GGGTTATTAATGATCTGTAG-3') and HIV3B2 (SEQ ID NO:39) followed by digestion with KpnI/EcoRI. This fragment was inserted into pBS-SK digested with KpnI and EcoRI to yield pBSMIDMN.

A 1,028 bp SalI/XbaI fragment was derived from pMN1.8–10 by PCR using oligonucleotides HIVMN5 (SEQ ID NO:83) (5'-ATCATCGAGCTCTGTTCCTTGGGTTCTTAG-3') and HIVMN3P (SEQ ID NO:84) (5'-ATCATCTCTAGAATAAAAATTATAGCAAAGCCCTTTCCAAGCC-3') followed by digestion with SacI and XbaI. This fragment was co-ligated into pBS-SK digested with EcoRI and XbaI with a 404 bp EcoRI/SacI fragment. The 404 bp fragment was derived by PCR with pMN1.8–9 as template and oligonucleotides HIV3B1 (SEQ ID NO:32) and HIVMN4 (SEQ ID NO:85) (5'-ATCATCGAGCTCCTATCGCTGCTC-3'). The resultant plasmid was designated as pBS3MN.

The 1,026 bp EcoRI/KpnI fragment from pBSMIDMN was inserted into the 4,315 bp pBS3MN digested with EcoRI/KpnI to generate pBSMID3MN. This plasmid contains most of the env gene except the 5'-most region. The vaccinia virus H6 promoter (Goebel et al., 1990a,b) and the 5'-most region of the env gene were obtained by isolating a 318 bp KpnI fragment from pBSH6HIV3B5P (defined in Example 2). This fragment was ligated into KpnI/XbaI digested PBS-SK along with the 2.9 bp KpnI/XbaI fragment from pBSMID3MN. The resultant plasmid was designated as pH6HMNE.

The 2.7 kb NruI/XbaI fragment from pH6HMNE, containing the entire HIV-1(MN) env gene juxtaposed 3' to the 3'-most 26 bp of the H6 promoter, was blunt-ended and inserted into NruI/SmaI digested pSPHAH6. This generated plasmid pHAHIVMNE. Plasmid pSPHAH6 was derived as follows. Plasmid pMP2VCL (containing a polylinker region within vaccinia sequences upstream of the K1L host range gene) was digested within the polylinker with HindIII and XhoI and ligated to annealed oligonucleotides SPHPRHA A through D (SPHPRHA A (SEQ ID NO:86) 5'-AGCTTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGAGGGT - 3'SPHPRHA B (SEQ ID NO:87) (5'-TGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTTCATTATCGCGATATCCGTTAAGTTTG TATCGTAC-3') SPHPRHA C (SEQ ID NO:88) (3'-TTATTAGTATTTAATAAAGTAATAGCGCTATAGGCAATTCAAACATAGCATGAGCT-5') SPHPRHA D (SEQ ID NO:89) (3'-AGAAATAAGATATGAATTTTTCACTTTTATTTATGTTTCCAAGAACTCCCAACACAATTTAA- CTTTCGCTCT-5') generating pSP126 containing a HindIII site, H6 promoter -124 through -1 (Perkus et al., 1989) and XhoI, KpnI, SmaI, SacI and EcoRI sites.

Plasmid pSD544 (containing vaccinia sequences surrounding the site of the HA gene replaced with a polylinker region and translation termination codons in six reading frames) was digested with XhoI within the polylinker, filled in with the Klenow fragment of DNA polymerase I and treated with alkaline phosphatase. SP126 was digested with HindIII, treated with Klenow and the H6 promoter isolated by digestion with SmaI. Ligation of the H6 promoter fragment to pSD544 generated pSPHAH6 which contained the H6 promoter in the polylinker region (in the direction of HA transcription). This insertion plasmid enables the replacement of the vaccinia HA gene (A56; Goebel et al., 1990a,b) with foreign genetic material.

The C5L insertion plasmid was derived as follows. Using the cosmid vector pVK102 (Knauf and Nester, 1982), a genomic library for vCP65 (ALVAC-based rabies G recombinant with rabies in C5 locus) was constructed. This library was probed with the 0.9 kb PvuII canarypoxvirus genomic fragment contained within pRW764.5 (C5 locus). These canarypox DNA sequences contain the original insertion locus. A clone containing a 29 kb insert was grown up and

designated pHCOS1. From this cosmid containing C5 sequences, a 3.3 kb Cla fragment was subcloned. Sequence analysis from this ClaI fragment was used to extend the map of the C5 locus from 1-1372.

The C5 insertion vector, pC5L, was constructed in two steps. The 1535 bp left arm was generated by PCR amplification using oligonucleotides C5A (SEQ ID NO:90) (5'-ATCATCGAATTCTGAATGTTAAATGTTATACTTTG-3') and C5B (SEQ ID NO:91) (5'-GGGGGTACCTTTGAGAGTACCACTTCAG-3'). The template DNA was canarypoxvirus genomic DNA. This fragment was cloned into EcoRI/SmaI digested pUC8. The sequence was confirmed by standard sequencing protocols. The 404 bp right arm was generated by PCR amplification using oligonucleotides C5C (SEQ ID NO:92) (5'-ATCATCCTGCAGGTATTCTAAACTAGGAATAGATG-3') and C5DA (SEQ ID NO:93) (5'-ATCATCCTGCAGGTATTCTAAACTAGGAATAGATG-3). This fragment was then cloned into the vector previously generated containing the left arm digested with SmaI/PstI. The entire construct was confirmed by standard sequence analysis and designated pC5L. This insertion plasmid enables the insertion of foreign genes into the C5 locus.

The 2.8 kb XbaI/partial KpnI fragment from pH6HMNE was isolated and inserted into pC5L digested with XbaI and KpnI. The resultant plasmid was designated as pC5HIVMNE.

Plasmids pHAHIVMNE and pC5HIVMNE were used in vitro recombination experiments with NYVAC (vP866) and ALVAC (CPpp), respectively, as the rescue virus. These were done by standard procedures (Piccini et al., 1987). Plaques derived from recombinant virus were identified by plaque hybridization using a radiolabeled env-specific DNA probe (Piccini et al., 1987). After three rounds of plaque purification, the recombinant viruses were amplified. The NYVAC-based HIV-1(MN) env recombinant was designated vP1008 and the ALVAC-based recombinant vCP125.

Recombinant viruses, vCP125 and vP1008, were analyzed for expression of the HIV-1(MN) env gene by immunofluorescence and immunoprecipitation using previously reported procedures (Taylor et al., 1990). Pooled human sera from HIV-seropositive individuals (obtained from Dr. K. Steimer, Chiron Corp., Emeryville, Calif.) was used in these assays. Results from immunofluorescence revealed that cells infected with either vCP125 or vP1008 express the HIV-1 (MN) gene product on their surface. Immunoprecipitation from lysates prepared from vP1008 and vCP125 infected cells demonstrated the presence of three predominant HIV-1-specific proteins with apparent molecular masses of 160 kDa, 120 kDa, and 41 kDa, respectively. These are consistent with expression of the precursor envelope glycoprotein (160 kDa) and the proteolytically derived mature forms (120 kDa and 41 kDa).

EXAMPLE 11

EXPRESSION OF THE HIV-1(MN) gp120 BY NYVAC AND ALVAC

A 391 bp EcoRI/XbaI fragment was amplified from pBS3MN using oligonucleotides T7 (SEQ ID NO:94) (5'-AATACGACTCACTATAG-3') and HIVMN120 (SEQ ID NO:95) (5'-ATCATCTCTAGAATAAAAATTATCTTTTTTCTCTCTGCACCACTC-3') followed by digestion with EcoRI and XbaI. This fragment was ligated to the 4.2 kb EcoRI/XbaI fragment derived from pH6HMNE (defined in Example 10). The resultant plasmid contains a poxvirus expression cassette for HIV-1(MN) gp120 in pBS-SK and was designated pBSHIVMN120.

A 1.7 kb XbaI/partial KpnI fragment was isolated and inserted into pC5L digested with KpnI/XbaI. The resultant plasmid was designated as pC5HIVMN120. The insertion plasmid for integrating the HIV-1(MN) gp120 gene into NYVAC was obtained by first isolating the 1.6 kb NruI/SmaI fragment from pBSHIVMN120. This fragment was inserted into pSPHAH6 digested with NruI and SmaI to provide pHAHIVMN120.

Insertion plasmids, pC5HIVMN120 and pHAHIVMN120, were used in recombination experiments with ALVAC (CPpp) and NYVAC (vP866) as the rescuing virus. These assays and plaque identification and purification were performed by standard procedures (Piccini et al., 1987). Hybridization analyses were performed with a radiolabeled HIV-1(MN) gp120-specific probe. Purified recombinants were amplified. The ALVAC-based HIV-1(MN) gp120 recombinant was designated as vCP124 and the NYVAC-based HIV-1(MN) gp120 recombinant as vP1004.

Cells infected with vCP124 and vP1004 were analyzed for the presence of the recombinant expressed HIV-1(MN) gp120 by immunofluorescence and immunoprecipitation. These assays were performed as previously described (Taylor et al., 1990) using a pooled human sera from HIV-seropositive individuals (obtained from K. Steimer, Chiron Corporation, Emeryville, Calif.). Results from these studies clearly indicated that cells infected with either vCP124 and vP1004 contained HIV-1(MN) gp120, whereas gp120 was not observed in uninfected cells and cells infected with parental viruses, ALVAC and NYVAC.

EXAMPLE 12

EXPRESSION OF A NON-CLEAVABLE FORM OF HIV-1 gp160 BY ALVAC AND NYVAC

In order to express a non-cleavable form of the HIV-1 (IIIB) gp160 an arginine to threonine mutation was engineered at amino acid 511 (Ratner et al., 1985) as was demonstrated by Guo et al. (1990). These modifications were made to decrease the shedding of gp120 from the surface of infected cells. These manipulations were performed as follows. A 376 bp PstI/XbaI fragment was obtained by first amplifying the sequences from pBSHIV3BEII (described in Example 2) using oligonucleotides HIV3B2A (SEQ ID NO:96) (5'-GAAATAATAAAACAATAATC-3') and HIVECB (SEQ ID NO:97) (5'-GCTCCTATTCCCACTGCAGTTTTTTCTCTCTGCAC-3') followed by digestion with PstI and XbaI. This fragment was ligated with a 1,061 bp PstI/XbaI fragment and a 4.5 kb EcoRI/XbaI fragment from pBSHIV3BEII to yield pBSHIV3BEEC.

The central region of the Hantaan virus S segment was generated by PCR using oligonucleotides T5HT3PPS (SEQ ID NO:98) (5'GTCCTGCAGGATGGAAAAGAATGCCCCAAGC-3') and HTS55PN (SEQ ID NO:99) (5'-GGGGGAGGCAAACTACCAAGG-3') and the S^- specific cDNA clone as template. The 581 bp fragment contains a PstI site at its 3' end and the 5' end includes the NciI site of position 499 of the S segment (Schmaljohn et al., 1986). Furthermore, using the oligonucleotide T5HT3PPS (SEQ ID NO:98) eliminates the T_5NT element at position 1029 to 1035 without altering the amino acid sequence. This fragment was then digested with NciI and PstI. The PCR

fragment containing the 5' end of the coding sequence fused to the H6 promoter (HindIII/NciI digested above) was ligated into pBS-SK digested with HindIII and PstI along with the 581 bp NciI/PstI fragment containing the central region of the S segment. The resultant plasmid was designated pBSHTSH65P.

The 3' most 438 bp of the S segment was derived by PCR using oligonucleotides HTS3PXBA (SEQ ID NO:100) (5'-ATCATCTCTAGAATAAAAATTAGAGTTTCAAAGGC-3') and T5HT5PSP (SEQ ID NO:101) (5'-CGCCAGCATGCAGAAGCAGC-3') and the S-specific cDNA clone as template. The 5' end of this fragment contains the PstI site situated at position 1039 of the S segment coding sequence (Schmaljohn et al., 1986) and the 3' end contains a T_5NT sequence motif and a unique XbaI prior to insertion into PstI/XbaI digested pBS-SK to yield pBSHTS3P.

To generate the entire S segment expression cassette, a 1122 bp PstI/partial HindIII fragment was derived from pBSHTSH56P. This fragment was co-inserted into HindIII/XbaI digested pBS-SK with a 290 bp PstI/XbaI fragment from pBSHTS3P. The resultant plasmid was designated pBSHVS by linearization with XbaI followed by a partial HindIII digestion.

The 2.6 kb NruI/XbaI fragment from pBSHIV3BEEC, containing the 3'-most 26 bp of the H6 promoter linked to the gp160 cassette, was isolated and ligated to a 3.0 kb NruI/XbaI fragment of pBSHVS to yield pBSHIV3BEECM. Digestion with NruI and XbaI excises the 3'-most 26 bp of the H6 promoter and the Hantaan virus S sequence. The 3.0 kb NruI/XbaI fragment contains the 5'-most 100 bp of the H6 promoter in a PBS-SK plasmid.

The 2.8 kb XbaI/partial KpnI fragment from pBSHIV3BEECM was ligated to XbaI/KpnI digested pC5L to yield pC5HIV3BEEC. A 2.7 kb NruI/XbaI fragment from pBSHIV3BEECM was blunt-ended with the Klenow fragment of the *E. coli* DNA polymerase and inserted into NruI/SmaI digested pSPHAH6 to yield pHAHIV3BEEC.

The insertion plasmids, pC5HIV3BEEC and pHAHIV3BEEC, were used in in vitro recombination experiments by standard procedures (Piccini et al., 1987) using ALVAC (CPpp) and NYVAC (vP866), respectively, as rescue virus. Recombinant plaques were identified by standard plaque hybridization analysis (Piccini et al., 1987) using a radiolabeled probe specific for the HIV-1 env gene. Recombinant viruses were amplified following three rounds of purification. The recombinant ALVAC-based HIV-1(IIIB) gp160 (non-cleavable) was designated as vCP126 and the NYVAC-based equivalent as vP1020.

Immunofluorescence and immunoprecipitation analyses were performed by previously described procedures on vP1020 and vCP126 infected cells using pooled human serum from HIV-seropositive individuals (obtained from K. Steimer, Chiron Corp., Emeryville, Calif.). Immunofluorescence results clearly demonstrated the surface expression of the HIV-1(IIIB) gp160 (non-cleavable form) on the surface of cells infected with either vCP126 or vP1020. Furthermore, immunoprecipitation results demonstrated the presence of a HIV-1(IIIB) gp160 in these infected cells that was not proteolytically cleaved into the mature gp120 and gp41 frames.

A non-cleavable form of the HIV-1(MN) gp160 was also pursued and the recombinant viruses obtained as follows.

A PstI/XbaI fragment was obtained by PCR amplification from pH6HMNE (described in Example 10) using oligonucleotides HIVMN3P (SEQ ID NO:102) (5'-ATCATCTCTAGAATAAAAATTATAGGAAAGCCCTTTCCAAGCC-3') and HIVECA (SEQ ID NO:103) (5'-GTGCAGAGAAAAAACTGCAGTGGGAATAGGAGC-3') followed by digestion with PstI and XbaI. This 1061 bp fragment was ligated with the 391 bp EcoRI/PstI from pBSHIVMNT (described in Example 13) and the 4.2 kb EcoRI/XbaI fragment from pH6HMNE (defined in Example 10). The resultant plasmid was designated as pBSHIVMNEEC[1]. Sequence analysis of the HIV env insert demonstrated that a single nucleotide was missing (between the SacI and XbaI sites). To correct this, the following manipulations were performed. The 1.028 kb SacI/XbaI from pH6HMNE was used to substitute for the corresponding fragment from pBSHIVMNEEC resulting in the formation of pBSHIVMNEEC.

The 2.6 NruI/XbaI fragment from pBSHIVMNEEC was isolated, blunt-ended with Klenow, and inserted into NruI/SmaI digested pSPHAH6 (defined in Example 10). The resultant plasmid was designated pHAHIVMNEEC. The 2.6 kb NruI/XbaI fragment from PBSHIVMNEEC was also inserted into NruI/XbaI digested pVQH6C5LSP6 (below) to yield pC5HIVMNEEC.

Insertion plasmids, pHAHIVMNEEC and pC5HIVMNEEC, were used in standard recombination experiments (Piccini et al., 1987) with NYVAC (vP866) and ALVAC (CPpp), respectively, as rescue virus. Recombinant virus was identified and plaque purified by standard plaque hybridization (Piccini et al., 1987) using a radiolabeled HIV env-specific DNA probe. Purified recombinant virus were then amplified. The NYVAC-based recombinant containing the HIV-1 (MN) non-cleavable gp160 was designated as vP1078 and the ALVAC equivalent vCP144.

Expression analysis of vCP126 and vP1078 was performed as described above. These results demonstrated that expression was qualitatively equivalent to the HIV-1(IIIB) counterparts, vP1020 and vCP126.

EXAMPLE 13

EXPRESSION OF A NON-CLEAVABLE, SECRETED FORM OF HIV-1 env BY ALVAC AND NYVAC

ALVAC- and NYVAC-based recombinant viruses were generated which express an HIV-1(MN) env that is not proteolytically cleaved and is secreted by virtue of the elimination of the transmembrane sequence near the carboxy terminus of the gene product. A 502 bp PstI/XbaI fragment was obtained by first amplifying these sequences from pH6HMNE (defined in Example 10) using oligonucleotides HIVECA (SEQ ID NO:103) and HIVMNT1 (SEQ ID NO:104) (5'-ATCATCTCTAGAATAAAAATTACAAACTTGCCCATTTATCCAATTCC-3') followed by digestion with PstI (5'-end) and XbaI (3'-end). This fragment corresponds to nucleotides 7219 to 7808 (Ratner et al., 1985). This fragment will serve as the 3'-end of the env expression cassette. As such, the env gene product will lack the transmembrane region, will be terminated by a termination codon provided by oligonucleotide HIVMNT1 (SEQ ID NO:104), and will not be cleaved due to an amino change at 511 (defined in Example 12) provided using oligonucleotide HIVECA (SEQ ID NO:103). This 502 bp fragment was ligated to the 391 bp EcoRI/PstI fragment derived by PCR from pH6HMNE using oligonucleotides HIV3B1 (SEQ ID NO:32) and HIVECB (SEQ ID NO:97), and the 4.2 kb EcoRI/XbaI fragment to pH6MNE. The resultant plasmid was designated pBSHIVMNT.

The 2.2 kb XbaI/partial KpnI fragment from pBSHIVMNT was isolated and inserted into pC5L digested with XbaI and KpnI. The resultant plasmid was designated as pC5HIVMNT. The NYVAC insertion plasmid was derived by isolating the 2.1 kb NruI/XbaI fragment from pBSHIVMNT. This fragment was then blunt-ended with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2 mM dNTPs and inserted into pSPHAH6 digested with NruI and SmaI to yield pHAHIVMNT.

The insertion plasmids, pC5HIVMNT and pHAHIVMNT, were used in standard recombination experiments (Piccini et al., 1987) with ALVAC (CPpp) and NYVAC (vP866), respectively, as the rescue virus. Recombinant virus was identified by standard plaque hybridization assays (Piccini et al., 1987) using a radiolabeled HIV env-specific probe. Recombinant virus was subjected to three rounds of purification prior to amplification. The ALVAC-based HIV-1(MN) env (non-cleavable; secreted) was designated as vCP120 and the NYVAC equivalent as vP994.

Immunoprecipitation analyses were performed as previously described (above) for vCP120 and vP994 infected cells using pooled human sera from HIV-seropositive individuals. Both vCP120 and vP994 expressed an HIV-1(MN) env-specific gene product with a molecular weight consistent with a non-cleavable, truncated gene product. Furthermore, immunoprecipitation of the cell-free medium from vCP120 and vP994 infected cell cultures indicated the secretion of this env gene product.

A similar construction was engineered for the HIV-1(IIIB) env. The following manipulations were performed to accomplish this. A 487 bp PstI/XbaI fragment was obtained by first amplifying these sequences from pBSH6HIV3B5P (defined in Example 2) using oligonucleotides HIVECA (SEQ ID NO:103) and HIV3BT (SEQ ID NO:105) (5'-ATCATCTCTAGAATAAAAATTACAAACTTGCCCA-TTTATCTAATTCC-3') followed by digestion with PstI and XbaI. A 397 bp EcoRI/PstI fragment was isolated from pBSHIV3BEEC and a 4.2 kb EcoRI/XbaI fragment was isolated from pH6HIIIBEM. These three fragments were ligated together to yield pBSHIV3BT1. Plasmid pH6HIIIBEM was derived from pBSHIV3BEII (defined in Example 2) by digestion with KpnI to liberate a second copy of the H6 promoter linked to the 5' portion of the HIV-1 (IIIB) env gene. The 5.4 kp KpnI was then religated to form pBSHIV3BEII.

The 2.1 kb and 2.9 kb fragments derived by HindIII/XbaI digestion of pBSHIV3BEECM were ligated to the 105 bp HindIII/XbaI fragment from pBSHIV3BT1 to yield pBSHIV3BT. This plasmid was digested with NruI and XbaI to excise a 2.1 kb fragment. This fragment was blunt-ended and inserted into pSPHAH6 digested with NruI and SmaI to generate pHAHIV3BT.

The plasmid pHAHIV3BT was used in recombination experiments, as above, with NYVAC (vP866) as the rescue virus. Recombinant virus was identified and purified as above and the resultant recombinant was designated as vP1036. This recombinant had all the expression characteristics noted above for vCP120 and vP994.

EXAMPLE 14

EXPRESSION OF HIV-1(MN) gp120 ANCHORED WITH A TRANSMEMBRANE SEQUENCE BY NYVAC AND ALVAC

To fuse the env region encoding the gp120 to the region encoding the hydrophobic transmembrane sequence, the following manipulations were performed. A 200 bp fragment corresponding to the 3'-most region of the gp120 coding sequence was derived by PCR from pH6HMNE (defined in Example 10) using oligonucleotides HIV3B1 (SEQ ID NO:32) and HIVMN18 (SEQ ID NO:106) (5'-GCCTCCTACTATCATTATGAATAATCTTTTTCTCTC-TG-3'). This fragment was fused by PCR to annealed oligonucleotides HIVTM1 (SEQ ID NO:107) (5'-TTATTCATAATGATAGTAGGAGGCTTGG-TAGGTTTAAGAATAGTTTTTGCTG-TACTCTCTGTAGT GAATAGAGTTAGGCAGGGATAA-3') and HIVTM2 (SEQ ID NO:108) (5'-TTATCCCTGCCTAACTCTATTCACTACAGAGAGTA-CAGCAAAAACTATTCTTAAACCTACCAAGC CTCCTACTATCATTATGAATAA-3') using oligonucleotides HIV3B1 (SEQ ID NO:32) and HIVTM3 (SEQ ID NO:109) (5'-ATCATCTCTAGAATAAAAATTATCCC-TGCCTAACTCTATTCAC-3'). Oligonucleotides HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ ID NO:108) correspond to nucleotides 7850 to 7934 (Ratner et al., 1985) and represent the region encoding the HIV env hydrophobic anchor sequence. Fusion with HIVTM3 (SEQ ID NO:109) engineers the 3'-end of the eventual cassette with a termination codon and a 3'XbaI site. The derived fragment was digested with EcoRI/XbaI and ligated to pH6HMNE digested with EcoRI and XbaI to yield pBSHIVMN120T.

The 1.7 bk NruI/XbaI fragment from pBSHIVMN120T, containing the 3'-most 26 bp of the H6 promoter and the entire HIV-1 cassette, was isolated and inserted into the 5.1 kb NruI/XbaI fragment from pVQH6C5LSP6 to derive pC5HIVMN120T. The plasmid pVQH6C5LSP6 was derived as follows.

pC5L (defined in Example 10) was digested within the polylinker with Asp718 and NotI, treated with alkaline phosphatase and ligated to kinased and annealed oligonucleotides CP26 (SEQ ID NO:110) (5'-GTACGTGACTAATTAGCTATAAAAAGGATCCGGTA-CCCTCGAGTCTAGAATCGATCCC- GGGTTTT TATGACTAGTTAATCAC-3') and CP27 (SEQ ID NO:111) (5'-GGCCGTGATTAACTAGTCAT- AAAAACCCGG-GATCGATTCTAGACTCGAGGGTACCGGATCCTTTT TATAGCTAATTAGTCAC-3') (containing a disabled Asp718 site, translation stop codons in six reading frames, vaccinia early transcription termination signal (Yuen and Moss, 1987), BamHI, KpnI, XhoI, XbaI, ClaI, and SmaI restriction sites, vaccinia early transcription termination signal, translation stop codons in six reading frames, and a disabled NotI site) generating plasmid pC5LSP.

pC5LSP was digested with BamHI and ligated to annealed oligonucleotides CP32 (SEQ ID NO:112) (5'-GATCTTAATTAATTAGTCATCAG-GCAGGGCGAGAACGAGACTATCT-GCTCGTTAATTAATTAGGT CGACG-3') and CP33 (SEQ ID NO:113) (5'-GATCCGTCGACCTAATTAATTAACGAG-CACATAGTCTCGTTCTCGCCCTGCCT-GATGACTAATTA ATTAA-3') to generate pVQC5LSP6.

The 1.7 kb NruI/XbaI fragment from pBSHIVMN120T was also blunt-ended and inserted into pSPHAH6 digested with NruI and SmaI. The resultant plasmid was designated as pHAHIVMN120T.

Insertion plasmids, pC5HIVMN120T and pHAHIVMN120T, were used in standard recombination experiments (Piccini et al., 1987) with ALVAC and NYVAC, respectively, as the rescue virus. Recombinant virus was identified and purified by standard plaque hybridization

(Piccini et al., 1987) using a radiolabeled HIV-1 gp120-specific DNA probe. The pure populations were amplified and the ALVAC-based anchored HIV-1(MN) gp120 recombinant was designated vCP138. The NYVAC-based equivalent was designated vP1035.

Immunofluorescence and immunoprecipitation analyses were performed by standard procedures (above) to evaluate expression of the HIV-1(MN) anchored gp120 in vP138 and vP1035 infected cells. The assays were performed using pooled human sera from HIV-seropositive individuals (obtained from Dr. K. Steimer, Chiron Corp., Emeryville, Calif.). Investigation of surface immunofluorescence indicated that vCP138 and vP1035 infected cells contained HIV-1(MN) gp120 in the plasma membrane. Significantly, the surface staining of vCP138 and vP1035 infected cells was enhanced compared to cells infected with recombinant viruses (i.e. vCP125, vCP124, vP1004, and vP1008) expressing gp160 or a non-anchored gp120. Results from immunoprecipitation analyses confirmed the expression of gp120 in vCP138 and vP1035 infected cells and that the expressed product was of the expected molecular mass.

EXAMPLE 15

GENERATION OF NYVAC/HIV-1 GAG (PROTEASE⁻) RECOMBINANT

Plasmid pSD542 (a NYVAC TK locus donor plasmid; see Example 5) was derived from plasmid pSD460 (Tartaglia et al., 1992) by forming vector plasmids pSD513 as described above in Example 7. The polylinker region in pSD513 was modified by cutting with PstI/BamHI and ligating to annealed synthetic oligonucleotides MPSYN288 (SEQ ID NO:114) (5'GGTCGACGGATCCT 3') and MPSYN289 (SEQ ID NO:115) (5'GATCAGGATCCGTCGACCTGCA 3') resulting in plasmid pSD542.

A plasmid, pHXB2D, containing human immunodeficiency virus type 1 (HIV-1) cDNA sequence was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by cloning the 1,625 bp BglII fragment of pHXB2D, containing the 5'-end of the gag gene, into the 4,075 bp BglII fragment of pSD542. The plasmid generated by this manipulation is called pHIVG2.

The 3'-end of the gag gene was then cloned downstream from the rest of the gag gene. This was accomplished by cloning a 280 bp ApaI-BamHI PCR fragment, containing the 3'-end of the gag gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2. This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID NO:116) (5'-TGTGGCAAAGAAGGGC-3') and HIVP6 (SEQ ID NO:117) (5'-TTGGATCCTTATTGTGACGAGGGGTC-3'). The plasmid generated by this manipulation is called pHIVG3.

The I3L promoter was then cloned upstream of the gag gene. This was accomplished by cloning the oligonucleotides, HIVL17 (SEQ ID NO:118) (5'-GATCTTGAGATAAAGTGAAAATATATATCATTATATTACAAAGTACAATTATTTAGGTTTAATCATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGAT-3') and HIVL18 (SEQ ID NO:119) (5'-CGATCTAATTCTCCCCCGCTTAATACTGACGCTCTCGCACCCATGATTAAACCTAAATAATTGTA CTTTGTAATATAATGATATATATTTTCACTTTATCTCAA-3'), encoding the vaccinia virus I3L promoter and the 5'-end of the gag gene, into the 5,540 bp partial BglII-ClaI fragment of pHIVG3. The plasmid generated by this manipulation is called pHIVG4.

pHIVG4 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP969.

Immunoprecipitation analysis was performed to determine whether vP969 expresses authentic HIV-1 gag precursor protein.

Lysates from the infected cells were analyzed for HIV-1 gag precursor expression using pooled serum from HIV-1 seropositive individuals (obtained from Chiron, Emeryville, Calif.). The sera was preadsorbed with vP866 infected Vero cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4° C. with the HIV-1 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV-1 seropositive individuals specifically precipitated the HIV-1 gag precursor protein from vP969 infected cells, but did not precipitate HIV-1-specific proteins from mock infected or NYVAC infected cells.

EXAMPLE 16

GENERATION OF NYVAC/HIV-1 gag/pol RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by cloning the 1,625 bp BglII fragment of pHXB2D, containing the 5'-end of the gag gene, into the 4,075 bp BglII fragment of pSD542 (defined in Example 15). The plasmid generated by this manipulation is called pHIVG2.

The 3'-end of the gag gene was then cloned into pHIVG2. This was accomplished by cloning a 280 bp ApaI-BamHI PCR fragment, containing the 3'-end of the gag gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2. This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID NO:116) and HIVP6 (SEQ ID NO:117). The plasmid generated by this manipulation is called pHIVG3.

The I3L promoter was then cloned upstream of the gag gene. This was accomplished by cloning the oligonucleotides, HIVL17 (SEQ ID NO:118) and HIVL18 (SEQ ID NO:119), encoding the vaccinia virus I3L promoter and the 5'-end of the gag gene, into the 5,540 bp partial BglII-ClaI fragment of pHIVG3. The plasmid generated by this manipulation is called pHIVG4.

The portion of the gag gene encoding p24, p2, p7 and p6 was then eliminated. This was accomplished by cloning the oligonucleotides, HIVL19 (SEQ ID NO:120) (5'-CTGACACAGGACACAGCAATCAGGTCAGCCAAAATTACTAATTTTTATCTCGAGGTCGACAGGAC CCG-3') and HIVL20 (SEQ ID NO:121) (5'-GATCCGGGTCCTGTCGACCTCGAGATAAAAATTAGTAATTTTGGCTGAC-

CTGATTGCTGTGTCCT GTGTCAG-3'), into the 4,450 bp partial PvuII-BamHI fragment of pHIVG4. The plasmid generated by this manipulation is called pHIVG5.

The remainder of the gag gene, as well as the pol gene, was then cloned downstream of the p17 "gene". This was accomplished by cloning the 4,955 bp ClaI-SalI fragment of pHXB2D, containing most of the gag gene and all of the pol gene, into the 4,150 bp ClaI-SalI fragment of pHIVG5. The plasmid generated by this manipulation is called pHIVG6.

Extraneous 3'-noncoding sequence was then eliminated. This was accomplished by cloning a 360 bp AflII-BamHI PCR fragment, containing the 3'-end of the pol gene, into the 8,030 bp AflII-BamHI fragment of pHIVG6. This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP7 (SEQ ID NO:122) (5'-AAGAAAATTATAGGAC-3') and HIVP8 (SEQ ID NO:123) (5'-TTGGATCCCTAATCCTCATCCTGT-3'). The plasmid generated by this manipulation is called pHIVG7.

pHIVG7 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP989.

Immunoprecipitation experiments with vP989 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to the gag precursor protein, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP989 infected cells.

EXAMPLE 17

GENERATION OF NYVAC/HIV-1 gag/pol AND env (gp120) RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7, as described above (see Example 16).

pHIVG7 was used in recombination experiments with vP921 as the rescuing virus to yield vP991.

Immunoprecipitation experiments with vP991 infected cells were performed as described above for the expression of the HIV gag precursor protein. No HIV-specific species were precipitated from mock infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP991 infected cells.

EXAMPLE 18

GENERATION OF NYVAC/HIV-1 gag/pol AND env (gp160) RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7, as described above (see Example 16).

pHIVG7 was used in recombination experiments with vP911 (above) as the rescuing virus to yield vP990.

Immunoprecipitation experiments with vP990 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP990 infected cells.

EXAMPLE 19

GENERATION OF NYVAC/HIV-1 p17, p24 RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence, was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing pHIVG5, as described above (see Example 16).

The 3'-end of the p24 "gene" was then cloned into pHIVG5. This was accomplished by cloning a 660 bp SalI-BamHI PCR fragment, containing the 3'-end of the p24 "gene", into the 4,450 bp SalI-BamHI fragment of pHIVG5. This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP25 (SEQ ID NO:124) (5'-AAAGTCGACCCATATCACCTAGAAC-3') and HIVP26 (SEQ ID NO:125) (5'-TTTGGATCCTTACAAAACTCTTGCCTTAT-3'). The plasmid generated by this manipulation is called pHIVG8.

The entomopox 42 kd promoter was then cloned upstream of the p24 "gene". This was accomplished by cloning the oligonucleotides, HIVL21 (SEQ ID NO:126) (5'-TCGAGCAAAATTGAAAATATATAATTACAATATAAAATGCCTATAGTGCAGAACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCA-3') and HIVL22 (SEQ ID NO:127) (5'-TTTAAAGTTCTAGGTGATATGGCCTGATGTACCATTTGCCCCTGGATGTTCTGCACTATAGGCATTTTATATTGTAATTATATATTTTCAATTTTGC-3'), encoding the entomopox 42 kd promoter and the 5'-end of the p24 "gene", into the 5,070 bp XhoI-NsiI fragment of pHIVG8. The plasmid generated by this manipulation is called pHIVG9.

pHIVG9 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP970.

Immunoprecipitation experiments with vP970 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. A protein species corresponding to p24 was precipitated, however, from lysates of vP970 infected cells.

EXAMPLE 20

GENERATION OF NYVAC/HIV-1 p17, p24 AND env (gp120) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence, was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG9 as described above (see Example 19).

pHIVG9 was used in recombination experiments with vP921 as the rescuing virus to yield vP973.

Immunoprecipitation experiments with vP973 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected Vero cells. Protein species corresponding to env and p24 were precipitated, however, from lysates of vP973 infected cells.

EXAMPLE 21

GENERATION OF NYVAC/HIV-1 p17, p24 AND env (gp160) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence, was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence

encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG9, as described above (see Example 19).

pHIVG9 was used in recombination experiments with vP911 as the rescuing virus to yield vP971.

Immunoprecipitation experiments with vP971 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected Vero cells. Protein species corresponding to env and p24 were precipitated, however, from lysates of vP971 infected cells.

EXAMPLE 22

GENERATION OF NYVAC/HIV-1 gag (PROTEASE⁻) AND env (TRUNCATED) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG9 as described above (see Example 19).

An H6-promoted truncated HIV-1 envelope gene was then inserted into pHIVG4. This was accomplished by cloning the *E. coli* DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of pHIVE10, containing an H6-promoted truncated HIV-1 envelope gene, into the filled-in BamHI site of pHIVG4. The plasmid generated by this manipulation is called pHIVGE11.

The plasmid pHIVE10 was derived by inserting a SacI/partial KpnI fragment from pBSHIV3BCDT1 into the multiple cloning region of pIBI25 (IBI, New Haven, Conn.). The plasmid pBSHIV3BCDT1 contains an H6 promoted cassette to express a severely truncated form of the HIV-1(IIIB) envelope (amino acid 1 to 447; Ratner et al., 1985). Expression of this cassette was evaluated to eliminate CD4 binding while retaining the V3 loop region and the T1 epitope.

To construct pBSHIV3BCDT1 the following manipulations were performed. A PCR-derived fragment of 200 bp was amplified from pBSH6HIV3B5P (defined in Example 2) using oligonucleotides HIV3B2A (SEQ ID NO:96) and HIVCD4A (SEQ ID NO:128) (5'-GCCTCCTACTATCATTATGAATAAACTGATGGGAGGGGCATAC-3'). This fragment was fused by PCR to annealed oligonucleotides HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ ID NO:108) using oligonucleotides HIV3B2A (SEQ ID NO:96) and HIVTM3 (SEQ ID NO:109). These manipulations create the 3'-end of the truncated env cassette by placing sequences encoding the HIV-1 env transmembrane anchor (amino acids 691 to 718; Ratner et al., 1985), a translation termination codon (TAA), and a 3' XbaI site. This PCR-fusion product was digested with EcoRI and XbaI to yield a 243 bp fragment. The fragment was ligated to the 4.5 bp EcoRI/XbaI fragment of pH6HIIIBE to generate pBSHIV3BCDT1.

pHIVGE11 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP979.

Immunoprecipitation experiments with vP979 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to env and the gag precursor proteins were precipitated, however, from lysates of vP979 infected cells.

EXAMPLE 23

GENERATION OF NYVAC/HIV-1 gag/pol AND env (TRUNCATED) RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7 as described above (see Example 16).

An H6-promoted truncated HIV-1 envelope gene was then inserted into pHIVG7. This was accomplished by cloning the *E. coli* DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of pHIVE10 (defined in Example 22), containing an H6-promoted truncated HIV-1 envelope gene, into the filled-in BamHI site of pHIVG7. The plasmid generated by this manipulation is called pHIVGE12.

pHIVGE12 was used in recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP978.

Immunoprecipitation experiments with vP978 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP978 infected cells.

EXAMPLE 24

GENERATION OF NYVAC/HIV-1 gag/pol AND env (gp120) RECOMBINANT

The sequence encoding the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7, as described above (see Example 16).

The I3L-promoted gag and pol genes were then inserted into a canary pox insertion vector. This was accomplished by cloning the 4,360 bp partial BglII-BamHI fragment of pHIVG7, containing the I3L-promoted gag and pol genes, into the BamHI site of pVQH6CP3L. The plasmid generated by this manipulation is called pHIVGE14.

The H6-promoted HIV-1(MN) envelope (gp120) gene was then inserted into pHIVGE14. This was accomplished by cloning the oligonucleotides, HIVL29 (SEQ ID NO:129) (5'-GGCCGCAAC-3') and HIVL30 (SEQ ID NO:130) (5'-TCGAGTTGC-3'), and the 1,600 bp NruI-NotI fragment of pBSHIVMN120, containing the H6-promoted gp120 gene, into the 11,500 bp NruI-XhoI fragment of pHIVGE14. The plasmid generated by this manipulation is called pHIVGE15.

The H6-promoted envelope (gp120) gene and the I3L-promoted gag and pol genes were then inserted into a vaccinia virus insertion vector. This was accomplished by cloning the 6,400 bp NotI-BamHI fragment of pHIVGE15, containing the H6-promoted gp120 gene and the I3L-promoted gag and pol genes, into the 4,000 bp NotI-BglII fragment of pSD542VCVQ. The plasmid generated by this manipulation is called pHIVGE16.

pHIVGE16 was used in in vitro recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP988.

Immunoprecipitation experiments with vP988 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC

infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP988 infected cells.

EXAMPLE 25

GENERATION OF NYVAC/HIV-1 gag/pol AND env (gp160) RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVGE16 as described above (see Example 24).

The gp120 gene was then replaced by the gp160 gene. This was accomplished by cloning the 2,600 bp NruI-NotI fragment of pH6HMNE, containing the entire HIV-1(MN) envelope (gp160) gene, into the 8,000 bp partial NruI-NotI fragment of pHIVGE16. The plasmid generated by this manipulation is called pHIVGE19.

pHIVGE19 was used in in vitro recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP1009.

Immunoprecipitation experiments with vP1009 infected cells were performed as described above for the expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP1009 infected cells.

EXAMPLE 26

GENERATION OF ALVAC/HIV-1 gag/pol AND env (GP120) RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVGE15, as described above (see Example 24).

pHIVGE15 was used in recombination experiments with ALVAC (CPpp) as the rescuing virus to yield vCP117.

Immunoprecipitation analysis was performed as described above but with CEF cell monolayers to determine whether vCP117 expresses authentic HIV-1 gag and env gene products.

Lysates from the infected cells were analyzed for HIV gag and env gene expression using serum from HIV-1 seropositive individuals (obtained from New York State Department of Health). The sera was preadsorbed with ALVAC infected CEF cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4° C. with the HIV-1 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV-1 seropositive individuals specifically precipitated the HIV-1 gag and env proteins from vCP117 infected cells, but did not precipitate HIV-1-specific proteins from mock infected or ALVAC infected cells.

EXAMPLE 27

GENERATION OF ALVAC/HIV-1 gag/pol AND env (gp160) RECOMBINANT

The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVGE15 as described above (see Example 24).

The gp120 gene was then replaced by the gp160 gene. This was accomplished by cloning the 2,600 bp NruI-NotI fragment of pH6HMNE, containing the entire HIV-1(MN) envelope (gp160) gene, into the 9,800 bp NruI-NotI fragment of pHIVGE15. The plasmid generated by this manipulation is called pHIVGE18.

The canary pox flanking arm deleted in the previous step was then cloned into pHIVGE18. This was accomplished by cloning the 1,500 bp NotI fragment of pHIVGE15, containing the C3 flanking arm, into the 12,400 bp NotI fragment of pHIVGE18. The plasmid generated by this manipulation is called pHIVGE20.

pHIVGE20 was used in recombination experiments with ALVAC (CPpp) as the rescuing virus to yield vCP130.

Immunoprecipitation analysis was performed with CEF cell monolayers as described above to determine whether vCP130 expresses authentic HIV-1 gag and env gene products.

Lysates from the infected cells were analyzed for HIV-1 gag and env gene expression using pooled serum from HIV-1 seropositive individuals (obtained from Chiron, Emeryville, Calif.). The sera was preadsorbed with ALVAC infected CEF cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4° C. with the HIV-1 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography. Human sera from HIV-1 seropositive individuals specifically precipitated the HIV-1 gag and env proteins from vCP130 infected cells, but did not precipitate HIV-1-specific proteins from mock infected or ALVAC infected cells.

EXAMPLE 28

GENERATION OF ALVAC/HIV-1 gag/pol RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG7 as described above (see Example 16).

The gag and pol genes were then cloned between canary pox flanking arms. This was accomplished by cloning the

4,400 bp SmaI-NotI fragment of pHIVG7, containing the I3L-promoted gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) (5'-GGCCAAAC-3') and HIV2L7 (SEQ ID NO:132) (5'-TCGAGTTT-3'), into the SmaI-XhoI site of pSPCP3L. The plasmid generated by this manipulation is called pHIVG24.

pHIVG24 was used in recombination experiments with ALVAC (CPpp) as the rescuing virus to yield vCP152.

Immunoprecipitation experiments with vCP152 infected cells were performed as described above for the expression of the HIV-1 env and gag proteins. No HIV-1-specific species were precipitated from mock infected or ALVAC infected cells. Protein species corresponding to the gag precursor protein, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vCP152 infected cells.

EXAMPLE 29

GENERATION OF ALVAC/HIV-1 gag/pol AND env (TRUNCATED) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG24 as described above (see Example 28).

pHIVG24 was used in recombination experiments with vCP120 as the rescuing virus to yield vCP155.

Immunoprecipitation experiments with vCP155 infected cells were performed as described above for the expression of the HIV-1 env and gag proteins. No HIV-1-specific species were precipitated from mock infected cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vCP155 infected cells.

EXAMPLE 30

GENERATION OF ALVAC/HIV-1 gag/pol AND env (gp120 WITH TRANSMEMBRANE ANCHOR) RECOMBINANT

A plasmid, pHXB2D, containing HIV-1 cDNA sequence was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVG24 as described above (see Example 28).

pHIVG24 was used in recombination experiments with vCP138 as the rescuing virus to yield vCP156.

Immunoprecipitation experiments with vCP156 infected cells were performed as described above for the expression of the HIV-1 env and gag proteins (CEF Cell monolayer). No HIV-1-specific species were precipitated from mock infected cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature cleavage products, were precipitated, however, from lysates of vCP156 infected cells.

Expression of HIV-1 gag-specific gene products either alone or in combination with env by vaccinia virus has been shown to lead to the production of non-infectious virus-like particles (Haffar et al., 1990; Hu et al., 1990). With this background it was investigated whether cells infected with ALVAC-based recombinant expressing HIV-1 gag-pol and env genes would also produce such particles. Furthermore, if these ALVAC-based recombinants were used to infect non-avian cells (i.e. Vero, MRC-5, etc.) then HIV-1 virus-like particles could be purified without any poxvirus virion contaminants.

To evaluate particle formation using Vero cells infected with vCP156, the following experiment was performed. Vero cells were infected at an m.o.i. of approximately 5 pfu/cell. After a 24 hr infection period, the supernatant was harvested and clarified by centrifugation at 2000 rpm for 10 min. The supernatant was then spun through filters which have a molecular weight cutoff of 30,000 kDa (Centricell 60, Polysciences, Inc., Warrington, Pa.). Thus, any smaller molecules would pass through these filters. The material retained by the filters was then analyzed by standard Western blot analysis (Maniatis et al., 1990) using pooled human serum from HIV-seropositive individuals (obtained from Dr. J. Conroy, New York State Department of Health). The results from the Western blot analysis demonstrated the presence of the major core protein p24 and the HIV-1(MN) anchored gp120 in the material retained by the filters. With the size exclusion noted above, the p24 would have passed through unless it was in a higher structural configuration (i.e. virus-like particles). Therefore, these results strongly suggest that HIV-1 virus-like particles containing the gp120 envelope component are produced in vCP156 infected cells.

EXAMPLE 31

EXPRESSION OF THE T1, T2, AND TH4.1 EPITOPES OF THE HIV-1 env GENE IN ALVAC AND NYVAC

Recombinant poxviruses vP1062 and vCP146 were generated to express the T1, T2, and TH4.1 epitopes of HIV-1 env (Hosmalin et al., 1991) as individual peptides.

Construction of plasmid p731T1. Plasmid pMPI3H contains the vaccinia I3L early/intermediate promoter element (Schmitt and Stunnenberg, 1988; Vos and Stunnenberg, 1988) in a pUC8 background. The promoter element was synthesized by polymerase chain reaction (PCR) using pMPVC1, a subclone of vaccinia HindIII I, as template and synthetic oligonucleotides MPSYN283 (SEQ ID NO:133) (5'- CCCCCCAAGCTTACATCATGCAGTGGTTAAAC -3') and MPSYN287 (SEQ ID NO:134) (5'- GATTAAACCTAAATAATTGT -3') as primers. DNA from this reaction was cut with HindIII and RsaI and a 0.1 kb fragment containing the promoter element was purified. A linker region was assembled by annealing complementary synthetic oligonucleotides MPSYN398 (SEQ ID NO:135) (5'- ACAATTATTTAGGTTAACTGCA -3') and MPSYN399 (SEQ ID NO:136) (5'- GTTAACCTAAATAATTGT -3'). The PCR-derived promoter element and the polylinker region were ligated with vector plasmid PUC8 which had been cut with HindIII and PstI. The resulting plasmid, pMPI3H, contains the I3L promoter region from positions -100 through -6 relative to the initiation codon, followed by a polylinker region containing HpaI, PstI, SalI, BamHI, SmaI and EcoRI sites. Cleavage with HpaI produces blunt ended DNA linearized at position -6 in the promoter.

A cassette containing the T1 peptide driven by the vaccinia I3L promoter was generated by ligating complementary oligonucleotides T1C (SEQ ID NO:137) (5'-TAATCATGAAACAAATTATTAATATGTGGCAAGAAGAGG- AAAAGCTATGTACGCTTGACT AGTTAATCACTCGAG -3') and T1N (SEQ ID NO:138) (5'- GATCCTCGAGTGATTAACTAGTCAAGCGTA-

CATAGCTTTTCCTACTTCTTGCCACATATT AATAATTTGTTTCATGATTA -3') to plasmid pMPI3H digested with HpaI and BamHI. This ligation reconstitutes the last 5 base pairs of the promoter, provides the complete coding sequence of the T1 peptide, and creates a XhoI site between the stop codon and BamHI site. This is plasmid p731T1. The sequence of the fragment was confirmed by nucleotide sequence analysis.

Construction of plasmid pH6T2. A cassette containing the T2 peptide driven by the vaccinia H6 promoter was generated in two steps: The H6 promoter through the EcoRV site was derived from a plasmid containing the synthetic H6 promoter (Perkus et al., 1989), using PCR and primers H6PCR1 (SEQ ID NO:157) and H6PCR2 (SEQ ID NO:205) (5'-TTAACGGATATCGCGATAATG-3') creating a 5' HindIII site. This 122 bp PCR-derived fragment was digested with HindIII and EcoRV followed by ligation to similarly digested pBS-SK+ (Stratagene, La Jolla, Calif.), generating plasmid pBSH6. Complementary oligonucleotides T2C (SEQ ID NO:139) (5'- ATCCGTTAAGTTTGTATCGTAATGCACGAAGATATTATTTCTTTGTGGGATCAATCTTTA AAATGACTAGTTAATCAG -3') and T2N (SEQ ID NO:140) (5'- GATCCTGATTAACTAGTCATTTTAAAGATTGATCCCACAAAGAAATAATATCTTCGTGCA TTACGATACAAACTTAACGGAT-3') which complete the 3' end of the H6 promoter from the EcoRV site, encode the T2 peptide and create a BamHI site at the 3' end of the gene were annealed then ligated to pBSH6 that was digested with EcoRV and BamHI. This plasmid was designated pH6T2 following confirmation of the fragment by nucleotide sequence analysis.

Construction of plasmid pVQ42KTH4.1. A cassette containing the TH4.1 peptide driven by the AmEPV 42K promoter was generated by sequential PCR reactions: the 107 bp 42K promoter with 5' PstI and SmaI sites was derived by PCR from plasmid p42KRABI, a plasmid containing the gene for the rabies glycoprotein under control of the 42K promoter, using primers 42KVQ1 (SEQ ID NO:141) (5'-AATTAATTAGCTGCAGCCCCGGGTCAAAAAAATATAAATG -3') and 42KVQ2 (SEQ ID NO:142) (5'- CCTTGTACTACTTCAATTACTCTATCCATTTTATATTGTAATTATATATTTTC). The sequence of the 107 bp promoter region of this PCR-derived fragment is (SEQ ID NO:143) 5'-TCAAAAAAATATAAATGATTCACCATCTGATAGAAAAAAAATTTATTGGGAAGAATAT GATAATATTTTGGGATTTCAAAATTGAAAATATATAATTACAATATAAA -3'. The 159 bp PCR-derived fragment was fused to the coding sequences of TH4.1 with a second PCR using this fragment and synthetic oligonucleotides encoding the TH4.1 peptide TH41C (SEQ ID NO:144) (5'- ATGGATAGAGTAATTGAAGTAGTACAAGGAGCTTATAGAGCTATTAGATGACTAGTTAAT CACTCGAGGATCC -3') and TH41N (SEQ ID NO:145) (5'- GGATCCTCGAGTGATTAACTAGTCATCTAATAGCTCTATAAGCTCCTTGTACTACTTCAA TTACTCTATCCAT -3') as template and primers 42KTH41 (SEQ ID NO:146) (5'- ATCATCGGATCCTCGAGTGATTAAACTAGTCATCTAATAGCTC -3') and 42KVQ1 (SEQ ID NO:141). This 210 bp PCR-derived fragment was extended in the 5' direction, incorporating a BamHI site at the 5' end using the fragment and synthetic oligonucleotides VQC (SEQ ID NO:147) (5'- TTAATCAGGATCCTTAATTAATTAGTTATTAGACAAGGTGAAAACGAAACTATTTGTAGC TTAATTAATTAGCTGCAGCCCGGG -3') and VQN (SEQ ID NO:148) (5'-CCCGGGCTGCAGCTAATTAATTAAGCTACAAATAGTTTCGTTTTCACCTTGTCTAATAAC TAATTAATTAAGGATCCTGATTAA -3') as template for a third PCR using primers 42KTH41 (SEQ ID NO:146) and BAMVQ (SEQ ID NO:149) (5'- TTAATCAGGATCCTTAATTAATTAGTTATTAGAC -3'). Subsequent nucleotide sequence analysis revealed an error in the sequence of oligonucleotide 42KTH41 (SEQ ID NO:146) such that an extra base (an A) was inserted after position 24 as indicated by the underline in the above sequence for 42KTH41. This was corrected with a final PCR employing the 272 bp fragment derived from the third PCR as template with primers BAMVQ (SEQ ID NO:149) and 42KTH41A (SEQ ID NO:150) (5'- ATCATCGGATCCTCGAGTGATTAACTAGTCATCTAATAGCTC -3'). After the final PCR, the cassette was to contain BamHI, PstI, and SmaI sites 5' to 42K-TH4.1 with XhoI and BamHI sites 3'. This 271 bp PCR-derived fragment was digested with BamHI and cloned into the BamHI site of pBS-SK+ (Stratagene, La Jolla, Calif.) generating plasmid pVQ42KTH4.1. Nucleotide sequence analysis of this plasmid confirmed that the sequence of the promoter and coding region was correct. However, a 3 bp deletion was revealed resulting in loss of the 3' BamHI site.

Construction of plasmid pT1T2TH4.1. These three cassettes were combined into a singular plasmid pT1T2TH4.1 such that I3L-T1 is opposite in orientation to the other two genes in the following manner: A 170 bp HindIII/XhoI fragment was isolated from p731T1 and ligated to similarly digested pH6T2 generating pT1T2. A 290 bp BamHI/SacI fragment from pVQ42KTH4.1 was ligated to similarly digested pT1T2, creating pT1T2TH4.1. The sequence of the insert was confirmed by nucleotide sequence analysis.

pC5LSP (defined in Example 14) was digested with EcoRI, treated with alkaline phosphatase and ligated to self-annealed oligonucleotide CP29 (SEQ ID NO:151) (5'-AATTGCGGCCGC-3'), digested with NotI and linear purified followed by self-ligation. This procedure introduced a NotI site to pC5LSP, generating pNC5LSP5.

Plasmid pSD550 was derived from pSD548 as follows. Plasmid pSD548 (Tartaglia et al., 1992) is a vaccinia vector plasmid in which the I4L ORF (Goebel et al., 1990a,b) is replaced by a cloning region consisting of BglII and SmaI sites. To expand the multicloning region, pSD548 was cut with BglII and SmaI and ligated with annealed complementary synthetic oligonucleotides 539A (SEQ ID NO:152) (5'-AGAAAAATCAGTTAGCTAAGATCTCCCGGGCTCGAGGGTACCGGATCCTGATTAGTTAATTTTTG T-3') AND 539B (SEQ ID NO:153) (5'-GATCACAAAAATTAACTAATCAGGATCCGGTACCCTCGAGCCCGGGAGATCTTAGCTAACTGATT TTTCT-3'). In the resulting plasmid, pSD550, the multicloning region contains BglII, SmaI, XhoI, KpnI and BamHI restriction sites.

The 602 bp XhoI fragment from pT1T2TH4.1 containing the genes for the epitopes driven by their respective promoters was cloned into donor plasmids pNC5LSP5 and pSD550 in their XhoI sites. Nucleotide sequence analysis was used to confirm the sequence and the orientation of the insert. The resulting plasmids pC5T1T2TH4.1 and pI4T1T2TH4.1 were used in in vitro recombination experiments with ALVAC and NYVAC to generate recombinant viruses vCP146 and vP1062, respectively. These recombinant viruses were demonstrated to contain the desired genes by hybridization of a specific DNA probe to viral plaques.

EXAMPLE 32

EXPRESSION OF TWO FUSION PEPTIDES CONTAINING THE T1, T2, AND TH4.1 EPITOPES OF HIV-1 env WITH AND WITHOUT A TRANSMEMBRANE ANCHOR DOMAIN FROM HIV-1 env

Recombinant poxviruses vP1060, vP1061, vCP154 and vCP148 were created to express a fusion peptide consisting

of the signal sequences from HIV-1 env coupled to sequences corresponding to the T1, T2, and TH4.1 epitopes of HIV-1 env by cleavable linker regions. vP1060 and vCP154 differ from vP1061 and vCP148 in that the former recombinant viruses express the fusion peptide along with sequences corresponding to the transmembrane region of HIV-1 env.

Both fusion peptides include the 51 amino acid N-terminal portion of HIV-1 (IIIB) env, residues 1–50 (plus initiating Met) based on Ratner et al. (1985). The amino acid sequence of this signal region (SEQ ID NO:154) is MKEQKTVAMRVKEKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVP. This is followed by the T1, T2, and TH4.1 epitopes (Hosmalin et al., 1991) separated from the signal, each other, and anchor sequence where present, by a cleavable linker region up to 5 amino acids in length. The amino acid sequence of this region of the peptide (SEQ ID NO:155) is [signal]-PFRKQIINMWQEVGKAMYA-PPFRK-HEDIISLWDQSLK-PPFRK-DRVIEVVQGAYRAIR-[PPFRK-anchor]. The anchor domain is a 28 amino acid transmembrane region of HIV-1 (IIIB) env, residues 691–718. The amino acid sequence of this region (SEQ ID NO:156) is LFIMIVGGLVGLRIVFAVLSVVNRVRQG-[stop].

For both versions of the fusion peptide, the H6 promoter and HIV-1 env signal sequences were derived by PCR from plasmid pBSH6HIV3B5P (defined in Example 2) using primers H6PCR1 (SEQ ID NO:157) (5'ACTACTAAGCTTCTTTATTCTATACTTAAAAAGTG-3') and PCRSIGT1 (SEQ ID NO:158) (5'- CATATTAATTTGTTTTCTAAAAGGAGGTACCCCATAATAGACTGTG -3'). This 314 bp PCR-derived fragment consists of a 5' HindIII site followed by the H6 promoter and coding sequences for the signal, linker, and the first 6 amino acids of the T1 peptide.

The remainder of the coding region for the construct without the transmembrane region was generated by PCR amplification of oligonucleotides T2T4A (SEQ ID NO:159) (5'- GCTCCTCCTTTTAGAAAACACGAAGATATTATTTCTTTGTGGGATCAATCTTTAAAACCTCCTTTTAGAAAAGATAGAGTAATTGAAGTAGTAC -3') and T2T4B (SEQ ID NO:160) (5'- GTACTACTTCAATTACTCTATCTTTTCTAAAAGGAGGTTTTAAAGATTGATCCCACAAAG AAATAATATCTTCGTGTTTTCTAAAAGGAGGAGC -3') using primers PCRT1T2 (SEQ ID NO:161) (5'- AAACAAATTATTAATATGTGGCAAGAAGTAGGAAAAGCTATGTACGCTCCTCCTTTTAGA AAACACGAAG -3') and PCRT4END (SEQ ID NO:162) (5'- ACTACTTCTAGATTATCTAATAGCTCTATAAGCTCCTTGTACTACTTCAATTACTC -3'). This 177 bp PCR-derived fragment encodes the T1 peptide, a linker region, the T2 peptide, another linker region, and the TH4.1 peptide, followed by a 3' XbaI site. This fragment was fused with the 314 bp PCR-derived fragment containing the promoter and signal sequences by PCR with primers H6PCR1 (SEQ ID NO:157) and PCRT4END (SEQ ID NO:162). Following digestion of this 473 bp PCR-derived fragment with HindIII and XbaI a fragment of 455 bp was isolated from an agarose gel, ligated to similarly digested PBS-SK, and the sequence of the insert verified by nucleotide sequence analysis. The resulting plasmid was designated pBST1T2TH4.1.

The remainder of the coding region of the version with the transmembrane anchor was generated by PCR amplification of oligonucleotides, T2T4A (SEQ ID NO:159) and T2T4B (SEQ ID NO:160), using primers PCRT1T2 (SEQ ID NO:161) and PCRT4TM (SEQ ID NO:163) (5'- TACTATCATTATGAATAATTTTCTAAAAGGAGGTCTAATAGCTCTATAAGCTCCTTGTAC TACTTCAATTACTC -3'), altering the 3' end to accommodate the transmembrane region. This 195 bp PCR-derived fragment was fused by PCR with oligonucleotides comprising the anchor, HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ ID NO:108) using primers PCRT1T2 (SEQ ID NO:161) and PCRT4END (SEQ ID NO:162). This 276 bp PCR-derived fragment was fused with the 314 bp PCR-derived fragment containing the promoter and signal sequences by PCR with primers H6PCR1 (SEQ ID NO:157) and PCRT4END (SEQ ID NO:162). Following digestion of this 572 bp PCR-derived fragment with HindIII and XbaI a fragment of 554 bp was isolated from an agarose gel, ligated to similarly digested pBS, and the sequence of the insert verified by nucleotide sequence analysis. The resulting plasmid was designated pBST1T2TH4.1A.

pC5LSP (defined in Example 14) was digested with BamHI and ligated to annealed oligonucleotides CP32 (SEQ ID NO:112) and CP33 (SEQ ID NO:113) to generate pVQC5LSP6. pVQC5LSP6 was digested with EcoRI, treated with alkaline phosphatase and ligated to self-annealed oligonucleotide CP29 (SEQ ID NO:151), digested with NotI and linear purified followed by self-ligation. This procedure introduced a NotI site to pVQC5LSP6, generating pNVQC5LSP7.

Both cassettes were placed individually between the XhoI and XbaI sites of insertion plasmid pNVQC5LSP7. These plasmids pC5ST1T1TH4.1 and pC5ST1T2TH4.1A were used to generate canarypoxvirus recombinants, vCP148 and vCP154 respectively. BamHI-SmaI fragments were excised from pC5ST1T1TH4.1 and pC5ST1T2TH4.1A and ligated to similarly digested pSD550 (defined in Example 31) generating plasmids pI4ST1T2TH4.1 and pI4ST1T1TH4.1A, respectively. These plasmids were used in recombination experiments with NYVAC as the rescuing virus resulting in recombinants vP1061 and vP1060, respectively. These recombinant viruses were demonstrated to contain the desired genes by hybridization of a specific DNA probe to viral plaques.

EXAMPLE 33

EXPRESSION OF THE HIV-1 nef GENE IN ALVAC, TROVAC, AND NYVAC

Recombinant poxviruses vP1084, vFP174, and vCP168 were generated expressing HIV-1 nef (MN) as follows:

The I3L promoter was derived by PCR from plasmid pMPI3H using primers I3PCR1 (SEQ ID NO:164) (5'-ATCATCGGATCCAAGCTTACATCATGCAGTGG -3') and PI3NEF2 (SEQ ID NO: 165) (5'- CGTTTTGACCATTTGCCACCCATGATTAAACCTAAATAATTGTACTTTG -3'). The coding region of nef was generated by PCR amplification of pMN1.8–10 (a clone of that portion of the MN genome between SstI sites at 7792 and 9595) using primers PI3NEF1 (SEQ ID NO:166) (5'-AGTACAATTATTTAGGTTTAATCATGGGTGGCAAATGGTCAAAACG -3') and PNEFBAM (SEQ ID NO:167) (5'- ATCATCGGATCCTAACACTTCTCTCTCCGG -3'). Fusion of the coding region with the promoter was accomplished by amplification of the previous PCR products using primers I3PCR1 (SEQ ID NO:164) and PNEFBAM (SEQ ID NO:167). Following digestion of this product with BamHI a fragment of 0.7 kb was isolated from an agarose gel and ligated to similarly digested pBS-SK+ (Stratagene, La Jolla, Calif.), generating

plasmid pI3NEF. It was at this point that sequence deviations were first observed. The sequence of the cassette was determined to differ from the published sequence (Gurgo et al., 1988, GenBank Accession Number M17449) due to deviations in plasmid pMN1.8–10 (provided by Dr. R. C. Gallo, NCI-NIH). These differences are summarized below relative to the published sequence.

Base #	Gurgo et al.	MN1.8–10	Result	Cassette
8834	T	T	silent	C (aa = Arg)
8863	G	A	Lys->Arg	A (aa = Lys)
8890	T	C	Pro->Leu	C (aa = Pro)
9028	A	G	Arg->Lys	G (aa = Arg)
9127	A	A	silent	G (aa = Gln)
9330–9331	GG	GGG	frameshift	GG

The two silent mutations in the cassette (at positions 8834 and 9127) were apparently errors in PCR. Since there is no effect on the encoded protein, the se were allowed to persist. The frameshift at 9930 results in a lengthened open reading frame more closely resembling other HIV-1 isolates. In keeping with the published size of nef from the MN isolate, this cassette required a fourth PCR to generate the truncated 3' end of the coding region.

Removal of the extra base at position 9930 was accomplished by PCR amplification of the insert in pI3NEF with primers I3PCR1 (SEQ ID NO:164) and PNEFFIX1 (SEQ ID NO:168) (5'- ATCATCGGATCCTAACACTTCTCTCTCCGGGTCATCCATCCATGCTGGCTCATAG -3') Following digestion of this 678 bp PCR-derived fragment with BamHI a fragment of 660 bp was isolated from an agarose gel and ligated to similarly digested pBS, generating plasmid pBSI3NEF. The insert was verified by nucleotide sequence analysis.

The 660 bp BamHI fragment from pBSI3NEF containing the nef gene was placed in the BamHI site of insertion plasmid PNVQC5LSP7 (defined in Example 32). The resulting plasmid pC5I3NEF was employed in a recombination into the C5 locus of ALVAC, generating the recombinant vCP168. The same 660 bp BamHI fragment was also placed in the BamHI site of insertion plasmid pSD550 (defined in Example 31), creating plasmid pI4I3NEF. A recombination with this plasmid with NYVAC generated recombinant vP1084.

An insertion plasmid for the F16 locos of TROVAC was derived in the following manner. A 7.3 kb NaeI/NdeI fragment was isolated from a plasmid containing the 10.5 kb HindIII fragment of fowlpox virus described by Tartaglia et al. (1990) and ligated to similarly digested pUC9 creating plasmid pRW866.

pUC9 was digested with PvuII and an EcoRI linker ligated between the PvuII sites creating plasmid pRW715. A cloning site flanked by fowlpox sequences was generated by PCR amplification of a portion of the 10.5 kb fragment with primers RW264 (SEQ ID NO:169) (5'- AATTAACCCGGGATCCAAGCTTCTAGCTAGCTAATTTTTATAGCGGCCGCTATAATCGTTAACTTATTAG -3') and RW267 (SEQ ID NO:170) (5'- GCTAGAAATCTCTTAGTTTTTATAGTTG -3'). An adjacent region was also amplified by PCR using primers RW266 (SEQ ID NO:171) (5'- GTTACATATGTACAGAATCTGATCATAG -3') and RW265 (SEQ ID NO:172) (5'- CTAGCTAGAAGCTTGGATCCCGGGTTAATTAATTAATAAAAAGCGGCCGCGTTAAAGTAG AAAAATG -3'). These PCR-derived fragments were fused by a third PCR using primers RW266 (SEQ ID NO:171) and RW267 (SEQ ID NO:170). The resulting PCR-derived fragment consisted of fowlpox sequences flanked by a 5' EcoRI site and a 3' NdeI site. Central in the fragment is a polycloning region containing SmaI, BamHI, and HindIII sites, flanked by NotI sites and translation stop codons in six reading frames. An early transcription termination signal (Yuen and Moss, 1987) is adjacent to the 3' NotI site. This PCR-derived fragment, digested with EcoRI and NdeI, was ligated to similarly digested pRW715 creating plasmid pRW864. An 11K promoted lac Z gene was excised from pAM1 by partial BamHI, total PstI digestion. This fragment was made blunt ended with Klenow polymerase and ligated to SmaI digested pRW864, creating pRW867A. The NotI fragment from pRW867A was made blunt ended with Klenow polymerase in the presence of dNTPs so that the NotI sites would be regenerated when ligated into an FspI site, and ligated to pRW866 which was partially digested with FspI such that the insertion was made corresponding to position 1955 described by Tartaglia et al. (1990). The resulting plasmid, pRW868, was then digested with NotI to remove the lac Z cassette, and ligated to the 66 bp polylinker from pRW864 which was excised by NotI digestion. The resulting plasmid was designated pRW673. An 81 bp SmaI fragment was derived from pVQ42KTH4.1 (defined in Example 31) and inserted into SmaI digested pRW873 generating plasmid pVQ873.

The nef cassette was excised from pBSI3NEF as a 684 bp HindIII fragment for insertion into pVQ873 followed by recombination into the F16 locus of TROVAC to generate vFP174.

EXAMPLE 34

EXPRESSION OF HIV-2 GENES IN NYVAC

Generation of NYVAC/HIV2 gag/pol recombinant. A plasmid, PISSYEGP, containing the human immunodeficiency virus type 2 (HIV2) gag and pol genes was obtained from Dr. G. Franchini (NCI-NIH). The gag and pol genes from this plasmid were cloned downstream of the I3L promoter and between vaccinia virus tk flanking arms. This was accomplished by cloning the 4,400 bp BstUI-BglII fragment of pISSYEGP, containing the HIV2 gag and pol genes, and the oligonucleotides, SIVL1 (SEQ ID NO:69) and HIV2L1 (SEQ ID NO:173) (5'-CGCCCATGATTAAACCTAAATAATTGTACTTTGTAATATAATGATATATATTTTCACTTTATCTC AC-3'), containing the I3L promoter, into the 4,070 bp XhoI-BglII fragment of pSD542 (defined in Example 15). The plasmid generated by this manipulation is called pHIV21.

Extraneous 3'-noncoding sequence was then eliminated. This was accomplished by cloning a 280 bp BclI-SmaI PCR fragment, containing the 3'-end of the pol gene, into the 8,100 bp BclI-SmaI fragment of pHIV21. This PCR fragment was generated from the plasmid, pISSYEGP, with the oligonucleotides, HIV2P2 (SEQ ID NO:174) (5'-ATGGCAGTTCATTGCAT-3') and HIV2P3 (SEQ ID NO:175) (5'-TTCCCGGGAGATCTCTATGCCATTTCTCCAT-3'). The plasmid generated by this manipulation is called pHIV22.

pHIV22 was used in recombination experiments with NYVAC (vP866) as the rescuing virus to yield vP1045.

Immunoprecipitation analysis was performed to determine whether vP1045 expresses authentic HIV2 gag gene products.

Lysates from the infected cells were analyzed for HIV2 gag expression using pooled serum from HIV2 seropositive

individuals (obtained from Dr. G. Franchini (NCI-NIH)). The sera was preadsorbed with vP866 infected Vero cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4° C. with the HIV2 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV2 seropositive individuals specifically precipitated the HIV2 gag precursor protein, as well as various intermediate and mature gag cleavage protein products, from vP1045 infected cells, but did not precipitate HIV2-specific proteins from mock infected or NYVAC infected cells.

EXAMPLE 35

GENERATION OF NYVAC/HIV2 gag/pol AND env (gp160) RECOMBINANT

A plasmid, pISSYEGP, containing the HIV2 gag and pol genes was obtained from Dr. G. Franchini (NCI-NIH). The gag and pol genes from this plasmid were cloned downstream of the I3L promoter and between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIV22 as described above (see Example 34).

pHIV22 was used in recombination experiments with vP920 as the rescuing virus to yield vP1047.

Immunoprecipitation experiments with vP1047 infected cells were performed as described above for the expression of the HIV2 gag proteins. No HIV2-specific species were precipitated from mock infected cells. Protein species corresponding to the HIV2 env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP1047 infected cells.

EXAMPLE 36

GENERATION OF NYVAC/HIV2 gag/pol AND env (gp120) RECOMBINANT

A plasmid, pISSYEGP, containing the HIV2 gag and pol genes was obtained from Dr. G. Franchini (NCI-NIH). The gag and pol genes from this plasmid were cloned downstream of the I3L promoter and between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIV22 as described above (see Example 34).

pHIV22 was used in recombination experiments with vP922 as the rescuing virus to yield vP1044.

Immunoprecipitation experiments with vP1044 infected cells were performed as described above for the expression of the HIV2 gag proteins. No HIV2-specific species were precipitated from mock infected cells. Protein species corresponding to the HIV2 env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP1044 infected cells.

EXAMPLE 37

EXPRESSION OF HIV2 GENES IN ALVAC

Generation of ALVAC/HIV2 gag/pol and env (gp160) recombinant. The plasmid, pBSH6HIV2ENV (defined in Example 4), contains the H6-promoted HIV2 env (gp160) gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV, containing the H6-promoted env gene, and the oligonucleotides, HIV2L4 (SEQ ID NO:176) (5'-GGTTG-3') and HIV2L5 (SEQ ID NO:177) (5'-AATTCAACCGC-3'), into the XhoI-EcoRI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

pHIV25 was used in recombination experiments with ALVAC (CPpp) as the rescuing virus to yield vCP153.

Immunoprecipitation analysis was performed as described above, but with CEF cell monolayers to determine whether vCP153 expresses authentic HIV2 gag and env gene products.

Lysates from the infected cells were analyzed for HIV2 gag and env gene expression using pooled serum from HIV2 seropositive individuals (obtained from Dr. G. Franchini (NCI-NIH)). The sera was preadsorbed with ALVAC infected CEF cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated overnight at 4° C. with the HIV2 seropositive human sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Human sera from HIV2 seropositive individuals specifically precipitated the HIV2 env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, from vCP153 infected cells, but did not precipitate HIV2-specific proteins from mock infected or ALVAC infected cells.

EXAMPLE 38

EXPRESSION OF SIV GENES IN NYVAC GENERATION OF NYVAC/SIV env (gp120-gp28) AND gag (PROTEASE⁻) RECOMBINANT

Immunoprecipitation analysis was performed to determine whether vP948 (defined in Example 5) expresses authentic SIV env and gag precursor proteins.

Lysates from the infected cells were analyzed for SIV env and gag precursor expression using serum from SIV seropositive macaques (obtained from Dr. G. Franchini (NCI-NIH)). The sera was preadsorbed with NYVAC (vP866) infected Vero cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4° C. Following this incubation period, the material was washed 4×with 1×buffer A. Lysates precleared with normal macaque sera and protein A-sepharose were then incubated overnight

at 4° C. with the SIV seropositive macaque sera bound to protein A-sepharose. After the overnight incubation period, the samples were washed 4×with 1×buffer A and 2×with a $LiCl_2$/urea buffer. Precipitated proteins were dissociated from the immune complexes by the addition of 2×Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling for 5 min. Proteins were fractionated on a 10% Dreyfuss gel system (Dreyfuss et al., 1984), fixed and treated with 1M Na-salicylate for fluorography.

Macaque sera from SIV seropositive individuals specifically precipitated the SIV gag precursor protein and the envelope glycoprotein from vP948 infected cells, but did not precipitate SIV-specific proteins from mock infected cells.

EXAMPLE 39

GENERATION OF NYVAC/SIV gag/pol RECOMBINANT

A plasmid, pSIVGAGSS11G, containing SIV_{MAC142} cDNA sequence was obtained from Dr. G. Franchini (NCI-NIH). The gag and pol genes from this plasmid were cloned downstream of the I3L promoter and between vaccinia virus tk flanking arms. This was accomplished by preparing plasmid pSIVG5 as described above (see Example 5).

pSIVG5 was used in recombination experiments with NYVAC (vP866) as the rescuing virus to yield vP1042.

Immunoprecipitation experiments with vP1042 infected cells were performed as described above for the expression of the SIV env and gag precursor proteins. No SIV-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to the gag precursor protein, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP1042 infected cells.

EXAMPLE 40

GENERATION OF NYVAC/SIV gag/pol AND env (gp120-gp41) RECOMBINANT

pSIVG5 (Example 5) was used in recombination experiments with vP1050as the rescuing virus to yield vP1071.

Immunoprecipitation experiments with vP1071 infected cells show expression of SIV genes.

EXAMPLE 41

GENERATION OF NYVAC/SIV gag/pol AND env (gp120-gp28) RECOMBINANT

pSIVG5 (Example 5) was used in recombination experiments with vP874 as the rescuing virus to yield vP943.

Immunoprecipitation experiments with vP943 infected cells were performed as described above for the expression of the SIV env and gag precursor proteins. No SIV-specific species were precipitated from mock infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were precipitated, however, from lysates of vP943 infected cells.

EXAMPLE 42

GENERATION OF NYVAC/SIV p16, p28 RECOMBINANT

Immunoprecipitation experiments with vP942 (Example 5) infected cells were performed as described above for the expression of the SIV env and gag precursor proteins. No SIV-specific species were precipitated from mock infected Vero cells. Protein species corresponding to p16 and p28 were precipitated, however, from lysates of vP942 infected cells.

EXAMPLE 43

GENERATION OF NYVAC/SIV p16, p28 AND env (gp120-gp28) RECOMBINANT

Immunoprecipitation experiments with vP952 (Example 5) infected cells were performed as described above for the expression of the SIV env and gag precursor proteins. No SIV-specific species were precipitated from mock infected Vero cells. Protein species corresponding to env and p16 and p28 were precipitated, however, from lysates of vP952 infected cells.

EXAMPLE 44

GENERATION OF NYVAC/SIV env (gp120-gp41) RECOMBINANT

The plasmid, pSIVEMVC, contains the H6-promoted SIV_{MAC142} envelope gene (in vitro selected truncated version). The region of the envelope gene containing the premature termination codon was cloned into pBSK+. This was accomplished by cloning the 1,120 bp ClaI-BamHI fragment of pSIVEMVC into the ClaI-BamHI site of pBSK+. The plasmid generated by this manipulation is called pSIV10.

The upstream termination codon, TAG, was then changed to the original CAG codon. This was accomplished by cloning the oligonucleotides, SIVL20 (SEQ ID NO:178) (5'-CTAGCTAAGTTAAGGCAGGGGTATAGGC-CAGTGTTCTCTTCCCCACCCTCT-TATTTCCAGCAGAC TCATACCCAACAG-3') and SIVL21 (SEQ ID NO:179) (5'-GTCCTGTTGGGTATGAGTCTGCTG-GAAATAAGAGGGTGGGGAAGAGAA-CACTGGCCTATACCCCT GCCTTAACTTAG-3'), into the 4,000 bp NheI-PpuMI fragment of pSIV10. The plasmid generated by this manipulation is called pSIV11.

The region containing the modified codon was then cloned back into pSIVEMVC. This was accomplished by cloning the 380 bp BglII-NheI fragment of pSIV11, containing the modified codon, into the 5,600 bp partial BglII-NheI fragment of pSIVEMVC. The plasmid generated by this manipulation is called pSIV12.

pSIV12 was used in in vitro recombination experiments with NYVAC (vP866) as the rescuing virus to yield vP1050.

Immunoprecipitation experiments with vP1050 infected cells were performed as described above for the expression of the SIV env and gag precursor proteins. No SIV-specific species were precipitated from mock infected or NYVAC infected Vero cells. A protein species corresponding to env was precipitated, however, from lysates of vP1050 infected cells.

EXAMPLE 45

EXPRESSION OF SIV GENES IN ALVAC

Generation of ALVAC/SIV gag/pol recombinant. A plasmid, pSIVGAGSS11G, containing SIV_{MAC}142 cDNA sequence was obtained from Dr. G. Franchini (NCI-NIH).

The gag and pol genes from this plasmid were cloned downstream of the I3L promoter and between vaccinia virus flanking arms. This was accomplished by initially preparing plasmid pSIVG5 as described above (see Example 5).

The gag/pol genes were then cloned between canary pox flanking arms. This was accomplished by cloning the 4,500 bp SmaI-NotI fragment of pSIVG5, containing the I3L-promoted gag/pol genes, into the SmaI-NotI site of pC5L (defined in Example 10). The plasmid generated by this manipulation is called pSIVGC13.

pSIVGC13 was used in recombination experiments with ALVAC (CPpp) as the rescuing virus to yield vCP172.

Immunoprecipitation experiments with vCP172 infected cells show expression of SIV genes.

EXAMPLE 46

EXPRESSION OF SIV GENES IN ALVAC

Generation of ALVAC/SIV env (gp120-gp41) and gag/pol Recombinant. A plasmid, pSIVGAGSS11G, containing simian immunodeficiency virus (SIV_{mac142}) cDNA sequence was obtained from Genoveffa Franchini (NCI-NIH). The gag/pol genes from this plasmid were cloned downstream of the I3L promoter and between vaccinia virus flanking arms. This was accomplished by cloning the 4,800 bp CfoI-TagI fragment of pSIVGAGSS11G, containing the gag/pol genes, and the oligonucleotides, SIVL1 (SEQ ID NO:69) and SIVL2 (SEQ ID NO:70), encoding the vaccinia virus I3L promoter, into the 4,070 bp XhoI-AccI fragment of pSD542VCVQ. The plasmid generated by this manipulation is called pSIVG1.

Extraneous 3'-noncoding sequence was then eliminated. This was accomplished by cloning a 1,000 bp BamHI-HpaI PCR fragment, containing the 3'-end of the pol gene, into the 7,400 bp partial BamHI-HpaI fragment of pSIVG1. (This PCR fragment was generated from the plasmid, pSIVGAGSS11G, with the oligonucleotides, SIVP5 (SEQ ID NO:71) and SIVP6 (SEQ ID NO:72)). The plasmid generated by this manipulation is called pSIVG4.

Sequencing analysis revealed that pSIVG4 contains a single base pair deletion within the pol gene. To correct this error the 2,320 bp BglII-StuI fragment of pSIVG1 was cloned into the 6,100 bp partial BglII-StuI fragment of pSIVG4. The plasmid generated by this manipulation is called pSIVG5.

The gag/pol genes were then cloned between canary pox flanking arms. This was accomplished by cloning the 4,500 bp SmaI-NotI fragment of pSIVG5, containing the I3L-promoted gag/pol genes, into the SmaI-NotI site of pC5L. The plasmid generated by this manipulation is called pSIVGC13.

The SIV env gene was then cloned into pSIVGC13. This was accomplished by cloning the *E. coli* DNA polymerase I (Klenow fragment) filled-in 2,750 bp partial BglII-XhoI fragment of pSIV12, containing the H6-promoted SIV env gene, into the SmaI site of pSIVGC13. The plasmid generated by this manipulation is called pSIVGC14.

pSIVGC14 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

Generation of ALVAC/SIV env (gp120-gp41) Recombinant. A plasmid, pSIVEMVC, contains the H6-promoted simian immunodeficiency virus envelope gene (in vitro selected truncated version). The region of the envelope gene containing the premature termination codon was cloned into pBSK+. This was accomplished by cloning the 1,120 bp ClaI-BamHI fragment of pSIVEMVC into the ClaI-BamHI site of pBSK+. The plasmid generated by this manipulation is called pSIV10.

The upstream termination codon, TAG, was then changed to the original CAG codon. This was accomplished by cloning the oligonucleotides, SIVL20 (SEQ ID NO:178) and SIVL21 (SEQ ID NO:179), into the 4,000 bp NheI-PpuMI fragment of pSIV10. The plasmid generated by this manipulation is called pSIV11.

The region containing the modified codon was then cloned back into pSIVEMVC. This was accomplished by cloning the 380 bp BglII-NheI fragment of pSIV11 into the 5,600 bp partial BglII-NheI fragment of pSIVEMVC. The plasmid generated by this manipulation is called pSIV12.

The env gene was then cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp NruI-Asp718 fragment of pSIV12, containing the H6-promoted env gene, into the 7,400 bp NruI-Asp718 fragment of pNVQH6C5SLP18. The plasmid generated by this manipulation is called pSIVGC15.

pSIVGC15 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

EXAMPLE 47

GENERATION OF HIV1 GENES IN ALVAC

Generation of ALVAC/HIV1 gag (+pro) (IIIB) and gp120 (+transmembrane) (MN) Recombinant. A plasmid, pHXB2D, containing human immunodeficiency virus type 1 (HIV1) cDNA sequence (IIIB) was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by cloning the 1,625 bp BglII fragment of pHXB2D, containing the 5'-end of the gag gene, into the 4,075 bp BglII fragment of pSD542VCVQ. The plasmid generated by this manipulation is called pHIVG2.

The 3'-end of the gag gene was then cloned into pHIVG2. This was accomplished by cloning a 280 bp ApaI-BamHI PCR fragment, containing the 3'-end of the gag gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2. (This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID NO:116) and HIVP6 (SEQ ID NO:117)). The plasmid generated by this manipulation is called pHIVG3.

The I3L promoter was then cloned upstream of the gag gene. This was accomplished by cloning the oligonucleotides, HIVL17 (SEQ ID NO:118) and HIVL18 (SEQ ID NO:119), encoding the vaccinia virus I3L promoter and the 5'-end of the gag gene, into the 5,540 bp partial BglII-ClaI fragment of pHIVG3. The plasmid generated by this manipulation is called pHIVG4.

The portion of the gag gene encoding p24, p2, p7 and p6 was then eliminated. This was accomplished by cloning the oligonucleotides, HIVL19 (SEQ ID NO:120) and HIVL20 (SEQ ID NO:121), into the 4,450 bp partial PvuII-BamHI fragment of pHIVG4. The plasmid generated by this manipulation is called pHIVG5.

The remainder of the gag gene, as well as the pol gene, was then cloned downstream of the p17 "gene". This was accomplished by cloning the 4,955 bp ClaI-SalI fragment of pHXB2D, containing most of the gag gene and all of the pol gene, into the 4,150 bp ClaI-SalI fragment of pHIVG5. The plasmid generated by this manipulation is called pHIVG6.

Extraneous 3'-noncoding sequence was then eliminated. This was accomplished by cloning a 360 bp AflII-BamHI

PCR fragment, containing the 3'-end of the pol gene, into the 8,030 bp AflII-BamHI fragment of pHIVG6. (This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP7 (SEQ ID NO:122) and HIVP8 (SEQ ID NO:123)). The plasmid generated by this manipulation is called pHIVG7.

The I3L-promoted gag and pol genes were then inserted into a canary pox insertion vector. This was accomplished by cloning the 4,360 bp partial BglII-BamHI fragment of pHIVG7, containing the I3L-promoted gag and pol genes, into the BamHI site of pVQH6CP3L. The plasmid generated by this manipulation is called pHIVGE14.

The H6-promoted HIV1 gp120 (+transmembrane) gene was then cloned into pHIVGE14. This was accomplished by cloning the 1,700 bp NruI-SmaI fragment of pC5HIVMN120T, containing the gp120 (+transmembrane) gene, into the 11,400 bp NruI-SmaI fragment of pHIVGE14. The resulting plasmid is called pHIVGE14T.

Most of the pol gene was then removed. This was accomplished by cloning a 540 bp ApaI-BamHI PCR fragment, containing the 3'-end of the HIV1 protease "gene", into the 10,000 bp ApaI-BamHI fragment of pHIVGE14T. (This PCR fragment was generated from the plasmid, pHIVG7, with the oligonucleotides, HIVP5 (SEQ ID NO:116) and HIVP37 (SEQ ID NO:180; 5'-AAAGGATCCCCCGGGTTAAAAATTTAAAGTGCAACC-3')). This manipulation removes most of the pol gene, but leaves the protease "gene" intact. The plasmid generated by this manipulation is called pHIV32.

pHIV32 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

EXAMPLE 48

GENERATION OF HIV1 GENES IN NYVAC

Generation of NYVAC/HIV1 gag (+pro) and gp120 (+transmembrane) Recombinant. A plasmid, pHXB2D, containing human immunodeficiency virus type 1 (HIV1) cDNA sequence (IIIB) was obtained from Dr. R. C. Gallo (NCI-NIH). The sequence encoding the 5'-end of the gag gene was cloned between vaccinia virus tk flanking arms. This was accomplished by cloning the 1,625 bp BglII fragment of pHXB2D, containing the 5'-end of the gag gene, into the 4,075 bp BglII fragment of pSD542VCVQ. The plasmid generated by this manipulation is called pHIVG2.

The 3'-end of the gag gene was then cloned into pHIVG2. This was accomplished by cloning a 280 bp ApaI-BamHI PCR fragment, containing the 3'-end of the gag gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2. (This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID NO:116) and HIVP6 (SEQ ID NO:117)). The plasmid generated by this manipulation is called pHIVG3.

The I3L promoter was then cloned upstream of the gag gene. This was accomplished by cloning the oligonucleotides, HIVL17 (SEQ ID NO:118) and HIVL18 (SEQ ID NO:119), encoding the vaccinia virus I3L promoter and the 5'-end of the gag gene, into the the 5,540 bp partial BglII-ClaI fragment of pHIVG3. The plasmid generated by this manipulation is called pHIVG4.

The portion of the gag gene encoding p24, p2, p7 and p6 was then eliminated. This was accomplished by cloning the oligonucleotides, HIVL19 (SEQ ID NO:120) and HIVL20 (SEQ ID NO:121), into the 4,450 bp partial PvuII-BamHI fragment of pHIVG4. The plasmid generated by this manipulation is called pHIVG5.

The remainder of the gag gene, as well as the pol gene, was then cloned downstream of the p17 "gene". This was accomplished by cloning the 4,955 bp ClaI-SalI fragment of pHXB2D, containing most of the gag gene and all of the pol gene, into the 4,150 bp ClaI-SalI fragment of pHIVG5. The plasmid generated by this manipulation is called pHIVG6.

Extraneous 3'-noncoding sequence was then eliminated. This was accomplished by cloning a 360 bp AflII-BamHI PCR fragment, containing the 3'-end of the pol gene, into the 8,030 bp AflII-BamHI fragment of pHIVG6. (This PCR fragment was generated from the plasmid, pHXB2D, with the oligonucleotides, HIVP7 (SEQ ID NO:122) and HIVP8 (SEQ ID NO:123)). The plasmid generated by this manipulation is called pHIVG7.

The I3L-promoted gag and pol genes were then inserted into a canary pox insertion vector. This was accomplished by cloning the 4,360 bp partial BglII-BamHI fragment of pHIVG7, containing the I3L-promoted gag and pol genes, into the BamHI site of pVQH6CP3L. The plasmid generated by this manipulation is called pHIVGE14.

The H6-promoted HIV1 envelope "gp120" gene (MN) was then inserted into pHIVGE14. This was accomplished by cloning the 1,600 bp NruI-NotI fragment of pBSHIVMN120, containing the H6-promoted envelope "gp120" gene, and the oligonucleotides, HIVL29 (SEQ ID NO:129) and HIVL30 (SEQ ID NO:130), into the 11,500 bp NruI-XhoI fragment of pHIVGE14. The plasmid generated by this manipulation is called pHIVGE15.

The H6-promoted HIV1 envelope "gp120" gene (MN) and the I3L-promoted gag and pol genes (IIIB) were then inserted into a vaccinia virus insertion vector. This was accomplished by cloning the 6,400 bp NotI-BamHI fragment of pHIVGE15, containing the H6-promoted HIV1 envelope "gp120" gene (MN) and the I3L-promoted gag and pol genes (IIIB), into the 4,000 bp NotI-BglII fragment of pSD542 (defined in Example 15). The plasmid generated by this manipulation is called pHIVGE16.

The "gp120" gene was then replaced by the "gp160" gene. This was accomplished by cloning the 2,600 bp NruI-NotI fragment of pH6HMNE (defined in Example 10), containing the entire HIV1 envelope gene (MN), into the 8,000 bp partial NruI-NotI fragment of pHIVGE16. The plasmid generated by this manipulation is called pHIVGE19.

The part of the env gene encoding gp41 was then replaced with the env transmembrane region. This was accomplished by cloning the 1,700 bp NruI-NotI fragment of pBSHIVMN120T, containing the H6-promoted gp120 (+transmembrane) gene, into the 8,500 bp partial NruI-NotI fragment of pHIVGE19. The resulting plasmid is called pHIVGE19T.

Most of the pol gene was then removed. This was accomplished by cloning a 540 bp ApaI-SmaI PCR fragment, containing the 3'-end of the HIV1 protease "gene", into the 7,000 bp ApaI-SmaI fragment of pHIVGE19T. (This PCR fragment was generated from the plasmid, pHIVG7, with the oligonucleotides, HIVP5 (SEQ ID NO:116) and HIVP37 (SEQ ID NO:180)). This manipulation removes most of the pol gene, but leaves the protease "gene" intact. The plasmid generated by this manipulation is called pHIV33.

pHIV33 is used in in vitro recombination experiments with NYVAC as the rescuing virus.

EXAMPLE 49

GENERATION OF HIV2 GENES IN ALVAC

Generation of ALVAC/HIV2 gag/pol Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example 4), contains

the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

The env gene was then removed. This was accomplished by cloning the oligonucleotides, HIV2L8 (SEQ ID NO:181; 5'-CGATAAACCGC-3') and HIV2L9 (SEQ ID NO:182; 5'-GGTTTAT-3'), into the 8,800 bp partial ClaI-SstII fragment of pHIV25. The plasmid generated by this manipulation is called pHIV27.

pHIV27 was used in in vitro recombination experiments with ALVAC as the rescuing virus to yield vCP190.

Generation of ALVAC/HIV2 env (gp160) Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example 4), contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183; 5'-GGGAAAG-3') and HIV2L11 (SEQ ID NO:184; 5'-GATCCTTTCCC-3'), into the 7,000 bp partial BamHI-SmaI fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

pHIV28 was used in in vitro recombination experiments with ALVAC as the rescuing virus to yield vCP188.

Generation of ALVAC/HIV2 gp120 Recombinant. A plasmid, pBSH6HIV2ENV, contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV (defined in Example 4) and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L. The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the 7,000 bp partial BamHI-SmaI fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then removed. This was accomplished by cloning the 360 bp PstI-XbaI fragment of pBSHIV2120B, containing the 3'-end of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ ID NO:185; 5'-CTAGAAAACCGC-3') and HIV2L13 (SEQ ID NO:186; 5'-GGTTTT-3'), into the 5,600 bp PstI-SstII fragment of pHIV28. The plasmid generated by this manipulation is called pHIV29.

pHIV29 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

Generation of ALVAC/HIV2 gag/pol and gp120 Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example 4), contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the 7,000 bp partial BamHI-SmaI fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then removed. This was accomplished by cloning the 360 bp PstI-XbaI fragment of pBSHIV2120B, containing the 3'-end of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ ID NO:185) and HIV2L13 (SEQ ID NO:186), into the 5,600 bp PstI-SstII fragment of pHIV28. The plasmid generated by this manipulation is called pHIV29.

The gp120 gene was then cloned into pHIV25. This was accomplished by cloning the 1,550 bp NruI-SacII fragment of pHIV29, containing the H6-promoted gp120 gene, into the 9,000 bp NruI-SacII fragment of pHIV25. The plasmid generated by this manipulation is called pHIV30.

pHIV30 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

Generation of ALVAC/HIV2 gp120 (+transmembrane) Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example 4), contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp

XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the 7,000 bp partial BamHI-SmaI fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then removed. This was accomplished by cloning the 360 bp PstI-XbaI fragment of pBSHIV2120B, containing the 3'-end of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ ID NO:185) and HIV2L13 (SEQ ID NO:186), into the 5,600 bp PstI-SstII fragment of pHIV28. The plasmid generated by this manipulation is called pHIV29.

The HIV1 env transmembrane region was then cloned onto the end of the gp120 gene. This was accomplished by cloning the 500 bp EcoRI fragment of pHIV31, containing the 3'-end of the gp120 gene and the env transmembrane region, into the 5,600 bp EcoRI fragment of pHIV29. (pHIV31 was derived by cloning a 500 bp PstI-XbaI PCR fragment, containing the 3'-end of the gp120 gene and the env transmembrane region, into the PstI-XbaI site of pIBI25. This PCR fragment was generated from the PCR fragment, PCRTM1, and the oligonucleotides, HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ ID NO:108), with the oligonucleotides, HIV2P14 (SEQ. ID NO:187; 5'-CAGAAGTAGCATATATGT-3') and HIVTM3 (SEQ ID NO:109). PCRTM1 was generated from the plasmid, pHIV28, with the oligonucleotides, HIV2P14 (SEQ ID NO:187) and HIV2P15 (SEQ ID NO:188; 5'-GCCTCCTACTATCATTATGAATAATCTCTTATGTC-TCCCTGGAGC-3')). The plasmid generated by this manipulation is called pHIV34.

pHIV34 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

Generation of ALVAC/HIV2 gag/pol and gp120 (+ transmembrane) Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example 4), contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the 7,000 bp partial BamHI-SmaI fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then removed. This was accomplished by cloning the 360 bp PstI-XbaI fragment of pBSHIV2120B (defined in Example 4), containing the 3'-end of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ ID NO:185) and HIV2L13 (SEQ ID NO:186), into the 5,600 bp PstI-SstII fragment of pHIV28. The plasmid generated by this manipulation is called pHIV29.

The HIV1 env transmembrane region was then cloned onto the end of the gp120 gene. This was accomplished by cloning the 500 bp EcoRI fragment of pHIV31, containing the 3'-end of the gp120 gene and the env transmembrane region, into the 5,600 bp EcoRI fragment of pHIV29. pHIV31 was derived by cloning a 500 bp PstI-XbaI PCR fragment, containing the 3'-end of the gp120 gene and the env transmembrane region, into the PstI-XbaI site of pIBI25 (IBI, New Haven, Conn.). This PCR fragment was generated from the PCR fragment, PCRTM1, and the oligonucleotides, HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ ID NO:108), with the oligonucleotides, HIV2P14 (SEQ ID NO:187) and HIVTM3 (SEQ ID NO:109). PCRTM1 was generated from the plasmid, pHIV28, with the oligonucleotides, HIV2P14 (SEQ ID NO:187) and HIV2P15 (SEQ ID NO:188). The plasmid generated by this manipulation is called pHIV34.

The gp120 (+ transmembrane) gene was then cloned into a plasmid containing the HIV2 gag/pol genes. This was accomplished by cloning the 2,700 bp ClaI fragment of pHIV34, containing the H6-promoted gp120 (+transmembrane) gene, into the 6,800 bp ClaI fragment of pHIV30. (pHIV30 was derived by cloning the 1,550 bp NruI-SacII fragment of pHIV29, containing the H6-promoted gp120 gene, into the 9,000 bp NruI-SacII fragment of pHIV25.) The plasmid generated by this manipulation is called pHIV35.

pHIV35 is used in in vitro recombination experiments with ALVAC as the rescuing virus.

EXAMPLE 50

EXPRESSION OF TWO FUSION PEPTIDES CONTAINING THE p24 EPITOPE OF HIV-1 gag FUSED TO THE T1 AND V3 LOOP EPITOPES OF HIV-1 env WITH AND WITHOUT THE SIGNAL DOMAIN FROM HIV-1 env

Two expression cassettes were generated by a series of polymerase chain reactions described below. These cassettes differ in that one version encodes the signal sequences of HIV-1 env fused to the epitopes whereas the other does not.

The version of the fusion peptide with the signal is preceded by the 51 amino acid N-terminal portion of HIV-1 (IIIB) env, residues 1–50 (plus initiating Met) based on Ratner et al. (1985) followed by a cleavable linker region. The amino acid sequence of this region is (SEQ ID NO:189) MKEQKTVAMRVKEKYQHLWRWGWRWGTMLLGM-LMICSATEKLWVTVYYGVP-PFRK. Both versions of the fusion peptide, contain an amino acid sequence based on the defined T-cell epitopes of p24, the V3 loop (MN), and T1 of HIV-1 (MN) env. The peptide is designed such that the epitopes are separated from each other and the signal where present by the sequence (SEQ ID NO:189) PPFRK. The sequence of this region of the peptide is (SEQ ID NO:190) [signal-PFRK]-GPKEPFRDYVDRFYK-PPFRK-VQINCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAH CNISRAK-PPFR-KQIINMWQEVEKAMYA. In the version lacking the signal sequence, the region indicated by [signal-PFRK] is replaced by an initiating methionine only.

For the cassette with the signal, the H6 promoter and signal were derived by PCR from plasmid pBST1T2TH4.1

(described above) using primers H6PCR1 (SEQ ID NO:157; 5'- ACTACTAAGCTTCTTTATTCTATACTTAAAAAGTG -3') and SIGPCR24 (SEQ ID NO:191; 5'-AGGTCCCTTCCTGAATGGAGGTACCCCATAATAGACTG-3'). The p24 epitope was fused to the signal sequence by PCR amplification of this 307 bp PCR-derived fragment and oligonucleotides P24A (SEQ ID NO:192; 5'-CCATTCAGGAAGGGACCTAAAGAACCTTTTAGAGATTATGTAGATAGATTTTATAAACCACCTTTTAGAAAA-3') and P24B (SEQ ID NO:193; 5'-TTTTCTAAAAGGTGGTTTATAAAATCTATCTACATAATCTCTAAAAGGTTCTTTAGGTCCCTTCCTGAATGG-3') using primers H6PCR1 and P24PCR (SEQ ID NO:194; 5'-GTACAATTAATTTGTACTTTTCTAAAAGGTGGTTTATAAAATC-3'). This 377 bp PCR-derived fragment consists of the H6 promoter coupled to coding sequences for the signal, the p24 epitope and the first 6 amino acids of the V3 loop.

For the cassette without the signal, the H6 promoter were derived by PCR from plasmid pH6T2 (described above) using primers H6PCR1 (SEQ ID NO:157) and H6P24 (SEQ ID NO:195; 5'-GGTGGTTTATAAAATCTATCT- ACATAATCTCTAAAAGGTTCTTTAGGTCCCATTACGATACAAAC TTAACGG-3'). The p24 epitope was fused to the promoter by PCR amplification of this 187 bp PCR-derived fragment and oligonucleotides P24A (SEQ ID NO:192) and P24B (SEQ ID NO:193) using primers H6PCR1 (SEQ ID NO:157) and P24PCR (SEQ ID NO:194). This 214 bp PCR-derived fragment consists of the H6 promoter coupled to coding sequences for the p24 epitope and the first 6 amino acids of the V3 loop.

Coding sequences for the V3 loop region were derived by PCR amplification of the SmaI fragment from plasmid pHIVGE16EV (described above) using primers V3PCR1 (SEQ ID NO:196; 5'-AAACCACCTTTTAGAAAAGTACAAATTAATTGTAC-3') and V3PCR2 (SEQ ID NO:197; 5'-CTGCTTACGGAACGGTGGTTTTGCTCTACTAATGTTACAATG-3'). The T1 epitope was joined to the coding region for the V3 loop by PCR amplification of this 171 bp PCR-derived fragment and oligonucleotides MNT1A (SEQ ID NO:198; 5'-CCACCGTTCCGTAAGCAGATAATAAACATGTGGCAAGAAGTAGAAAAAGCTATGTATGCTTAA-3') and MNT1B (SEQ ID NO:199; 5'-TTAAGCATACATAGCTTTTTCTACTTCTTGCCACATGTTTATTATCTGCTTACGGAACGGTGG-3') as template using primers V3PCR1 (SEQ ID NO:196) and T1MNPCR (SEQ ID NO:200; 5'-TCATCAAAGCTTCTCGAGAAAAATTAAGCATACATAGCTTTTTC-3'). This 239 bp PCR-derived fragment consists of the last codon of p24 epitope, the V3 loop, ant the T1 epitope followed 3' by an early transcription termination signal (TTTTTNT) and XhoI and HindIII sites.

For the cassette with the signal, the promoter, signal and p24 epitope coding sequences were joined to the coding region for the V3 loop/T1 epitope by PCR amplification of the 377 bp and 239 bp PCR-derived fragments using primers H6PCR1 (SEQ ID NO:157) and T1MNPCR (SEQ ID NO:200). Following digestion of this 581 bp PCR-derived fragment with HindIII, a fragment of 563 bp was isolated from an agarose gel, ligated to similarly digested pBS (Stratagene, La Jolla, Calif.) creating plasmid pMNT1P24. The insert was verified by nucleotide sequence analysis.

For the cassette without the signal, the promoter and p24 coding sequences were joined to the coding region for the V3 loop/T1 epitope by PCR amplification of the 171 bp and 239 bp PCR-derived fragments using primers H6PCR1 (SEQ ID NO:157) and T1MNPCR (SEQ ID NO:200). Following digestion of this 418 bp PCR-derived fragment with HindIII, a fragment of 400 bp was isolated from an agarose gel, ligated to similarly digested pBS (Stratagene, La Jolla, Calif.) creating plasmid pMN24EV3T1NSA. The insert was verified by nucleotide sequence analysis.

A C6 insertion vector containing 370 bp upstream of C6, polylinker containing SmaI, PstI, XhoI, and EcoRI sites, and 1156 bp of downstream sequence was derived in the following manner. The 0.4 kb upstream sequence was generated by PCR amplification of a cosmid clone derived from purified genomic canarypox DNA using oligonucleotides C6A1SG (SEQ ID NO:201; 5'- ATCATCGAGCTCGCGGCCGCCTATCAAAAGTCTTAATGAGTT -3') and C6B1SG (SEQ ID NO:202; 5'- GAATTCCTCGAGCTGCAGCCCGGGTTTTTATAGCTAATTAGTCATTTTTTCGTAAGTAAGT ATTTTTATTTAA -3'). The 1.2 kb downstream arm was generated by PCR amplification of the same template using oligonucleotides C6C1SG (SEQ ID NO:203; 5'- CCCGGGCTGCAGCTCGAGGAATTCTTTTTATTGATTAACTAGTCAAATGAGTATATATAAT TGAAAAAGTAA -3') and C6D1SG (SEQ ID NO:204; 5'- GATGATGGTACCTTCATAAATACAAGTTTGATTAAACTTAAGTTG -3'). These fragments were fused by a third PCR employing gel purified 0.4 and 1.2 kb fragments as template for primers C6A1SG (SEQ ID NO:201) and C6D1SG (SEQ ID NO:204). The resulting 1.6 kb fragment was isolated from an agarose gel, digested with SacI and KpnI and ligated to similarly digested pBS (Stratagene, La Jolla, Calif.) generating C6 insertion plasmid pC6L.

Both expression cassettes were excised by PstI/XhoI digestion of pMNT1P24 and pMN24EV3T1NSA, isolated from agarose gels and ligated separately to similarly digested pC6L creating plasmids pC6P24FS and pC6P24FNS respectively, for recombination into the C6 locus of ALVAC. The resulting recombinants are designated vCP189 and vCP195, respectively.

BamHI/XhoI fragments from pMNT1P24 and pMN24EV3T1NSA were ligated to similarly digested pSD550VC (defined in Example 33) creating pI4P24FS and pI4P24FNS for recombination into the I4 locus of NYVAC generating vP1117 and vP1110, respectively.

Expression of Tetanus Toxin Fragment C in Poxviruses Expressing HIV-1 Proteins. It has been proposed that the addition of various Th epitopes from homologous (Good et al., 1987) and heterologous proteins (Francis et al., 1987) may be capable of recruiting T-cell help for specific B cell responses to synthetic peptide vaccines. In an effort to elicit enhanced immune responses to HIV-1 antigens, various T-cell epitopes derived from HIV-1 have been incorporated into recombinant poxviruses. To further pursue this strategy, tetanus toxin fragment C, which contains other known human T-helper cell epitopes (Ho et al., 1990), will be co-expressed with HIV-1 antigens in an ALVAC recombinant. The presence of these epitopes from tetanus toxin may enhance the immune response against HIV-1 by providing nonspecific T-cell help.

PstI/SmaI digested pC6P24FS (described above) was modified by ligating the PstI/SmaI fragment from plasmid pVQ42KTH4.1 (described above) creating pC6P24FSVQ. This plasmid was digested with NruI within the H6 promoter and XhoI at the 3' end and ligated to a 1.4 kb fragment isolated from similarly digested pH6TETC (described above). The resulting plasmid, pC6VQTETC, was con-

firmed by restriction digestion and nucleotide sequence analysis of the regions surrounding the cloning sites. Following confirmation pC6VQTETC was employed in recombination experiments with vCP112, vCP125 and vCP156.

EXAMPLE 51

PERIPHERAL BLOOD MONONUCLEAR CELL STUDIES WITH NYVAC/HIV-1 AND ALVAC/HIV-1 RECOMBINANTS

While broad issues concerning the immunogenicity of NYVAC/HIV-1 and ALVAC/HIV-1 constructs in man are best addressed within the context of clinical trials, a number of relevant insights have already been gleaned by in vitro cellular response assays. A central question regarding the use of these vaccine constructs in man is their capacity to impact on cellular anti-HIV-1 reactivities, especially cytotoxic T-lymphocytes (CTL) responses. Whether as a component of a preventative or a therapeutic vaccine strategy, there are ample precedents suggesting a beneficial role for CTL in a number of viral infections in man (McMichael et al., 1983; Moss et al., 1978; Borysiewicz et al., 1988). Although the precise contribution of anti-HIV-1 CTL in preventing or controlling viral infection remains to be elucidated, studies by Letvin and co-workers using the SIV/macaque animal model suggest that anti-SIV-1 CTL, especially gag-specific CTL, may represent a major determinant controlling disease progression (Letvin and King, 1990). This coupled with the observations of numerous investigators that anti-HIV-1 CTL activities can be measured directly from fresh peripheral blood mononuclear cells (PBMC) in a relatively high proportion of asymptomatic patients (reviewed in Walker and Plata, 1990; Autran et al., 1991), support the contention that elicitation of anti-HIV-1 CTL reactivities should be included as a major goal of both preventative and therapeutic vaccines.

To test the capacity of NYVAC/HIV-1 and ALVAC/HIV-1 constructs to impact on relevant CTL reactivities, a series of experiments were conducted involving a cohort of HIV-1 infected patients devoid of detectable direct anti-HIV-1 gp160 CTL reactivities. PBMCs were obtained from these patients and a portion of the PBMC were infected with either the fully replication-competent vaccinia/gp 160 construct vPE16 (Walker et al., 1987), or the replication-attenuated NYVAC/gp160 or ALVAC/gp160 constructs. The acutely infected PBMC were washed and used as stimulators for the remaining PBMC in a 10-day in vitro stimulation protocol. Controls included both unstimulated and control vector (i.e. parental poxvirus minus HIV-1 genes) stimulated cultures. Following the 10-day incubation in the absence of exogenous IL-2, cells were washed and evaluated for CTL activities against autologous B-lymphocyte cell lines (BLCL) infected with either a control vaccinia virus, vSC8, or the vaccinia/gp160 vPE16 construct. Additionally, phenotypic depletion of CD8+ cells was performed in parallel using magnetic microsphere sorting. The results of these studies shown in FIG. 11 revealed that whereas unstimulated or control vector-stimulated cultures had no detectable CTL activity against HIV-1 gp160 targeted BLCL, cultures stimulated with any of the HIV-1 gp160 constructs had a high level of anti-gp160 cytolytic activity, all of which was abolished following removal of CD8+ cells and thereby consistent with the appearance of CTL activities. Perhaps the most significant observation to come out of these studies was the consistent finding that the magnitude of the CTL response was greater in the NYVAC/gp160 and ALVAC/gp160-stimulated cultures than in those which were stimulated with the fully replication competent vPE16 infected PBMC. It is not known whether this apparent enhancement in CTL stimulation by NYVAC/gp160 and ALVAC/gp160 was due to differences in expression by the recombinants as compared to vPE16 or to the attenuation characteristics of the NYVAC and ALVAC vectors.

Extensive flow cytometric analyses of the various stimulated cultures was also performed in order to more thoroughly identify the effector cell phenotype which was elicited. When compared to either unstimulated or control vector stimulated cultures, generation of anti-gp160 cytolytic reactivities generally resulted in a decline in $CD3^+/CD4^+$ subpopulations and a compensatory rise in $CD3^+/CD8^+$ cells. Significant increases in $CD3^+/CD25^+$, $CD3^+/HLA\text{-}DR^+$, $CD8^+/S6F1^+$, $CD8^+/CD38^+$, and $CD3^+/CD69^+$ cells were also noted. When the cytolytic activities of each culture were plotted against increases in particular cell subpopulations, a linear relationship was found between cellular anti-gp160 reactivity and increases in the $CD8^+/S6F1^+$ population. This cellular phenotype broadly defines CTL (Morinoto et al., 1987).

These early pre-clinical studies with cells from HIV-1 infected patients clearly illustrate the capacity of NYVAC and ALVAC/HIV-1 vectors to elicit potent CTL activities from precursor populations which are contained within the PBMC pool of patients. Furthermore, these studies strongly exemplify the potential clinical utility of these vectors in the context of a therapeutic vaccine strategy. This could take the form of simple immunization with these vectors or could involve an ex vivo component of either cellular targeting and re-infusion or ex vivo CTL generation followed by large scale adoptive transfer. In either case, the combined safety of the non-replicating vectors and their inherently strong cellular immunogenicity make them ideal candidates for immune-based therapy in man.

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to the particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

REFERENCES

1. Autran, B., Plata, F. and Debre, P., J. AIDS, 4, 361–367 (1991)
2. Behbehani, A. M., Microbiological Reviews 47, 455–509 (1983).
3. Bertholet, C., Drillien, R. and Wittek, R., Proc. Natl. Acad. Sci. 82, 2096–2100 (1985).
4. Borysiewicz, L., Graham, S., Hickling, J., Mason, P. D., and Sissons, J. P. G., Eur. J. Immunol. 18, 269–275 (1988)
5. Chakrabarti, S., Robert-Guroff, M., Wong-Staal, F., Gallo, R. C., and Moss, B., Nature 320, 535–537 (1986).
6. Cianciolo, G. J., Copeland T. D., Oroszlan S., and Snyderman, R., Science 230, 453–456 (1985).
7. Clewell, D. B., and Helinski, D. R., Proc. Natl. Acad. Sci. USA 62, 1159–1166 (1969).
8. Clewell, D. B., J. Bacteriol 110, 667–676 (1972).
9. Colinas, R. J., Condit, R. C., and Paoletti, E., Virus Research 18, 49–70 (1990).
10. Derosiers, R. C., M. S., Wyand, T. Kodama, T. J. Ringler, L. O. Arthur, P. K. Seghal, N. L. Letvin, N. W. King, and M. D. Daniel Proc. Natl. Acad. Sci. USA 86, 6353–6357 (1989).
11. Dreyfuss, G., Adam, S. A., and Choi, Y.-D., Mol. Cell. Biol. 4, 415–423 (1984).

12. Engelke, D. R., Hoener, P. A., and Collins, F. S., Proc. Natl. Acad. Sci. USA 85, 544–548 (1988).
13. Franchini, G., Gurgo, C., Guo, H.-G., Gallo, R. C., Collati, E., Fargnoli, K. A., Hall, L. F., Wong-Staal, F., and Reitz, Jr., M. S., Nature (London) 328, 539–543 (1987).
14. Franchini, G., Fargnoli, K. A., Giomnini, F., Jagodzinski, L., DeRossi, A., Bosch, M., Biberfield, G., Fenyo, E. M., Albert, J., Gallo, R. C., and Wong-Staal, F., Proc. Natl. Acad. Sci. USA 86, 2433–2437 (1989).
15. Francis, M. J., Hastings, G. Z., Syred, A. D., McGinn, B., Brown, F., and Rowlands, D. J., Nature 330, 168–170 (1987).
16. Gillard, S., Spehner, D., Drillien, R., and Kirn, A., Proc. Natl. Acad. Sci. USA 83, 5573–5577 (1986).
17. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E., Virology 179, 247–266 (1990a).
18. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E., Virology 179, 517–563 (1990b).
19. Good, M. F., Maloy, W. L., Lunde, M. N., Margalit, H., Cornette, J. L., Smith, G. L., Moss, B., Miller, L. H., and Berzofsky, J. A., Science 235, 1059–1062 (1987).
20. Guo, H.-G., Veronese, F., Tschachler, E., pal, R., Kalyanaraman, V. S., Gallo, R. C., and Reitz, Jr., M. S., Virology 174, 217–214 (1990).
21. Guo, P., Goebel, S., Davis, S., Perkus, M. E., Taylor, J., Norton, E., Allen, G., Lanquet, B., Desmettre P., and Paoletti, E., J. Virol. 64, 2399–2406 (1990).
22. Guo, P., Goebel, S., Davis, S., Perkus, M. E., Lanquet, B., Desmettre, P., Allen, G., and Paoletti, E., J. Virol. 63, 4189–4198 (1989).
23. Gurgo, C., Guo, H.-G., Franchini, G., Aldovini, A., Collalti, E., Farrell, K., Wong-Staal, F., Gallo, R. C., and Reitz, M. S., Jr., Virology 164, 531 (1988).
24. Haffar, O., Garrigues, J., Travis, B., Moran, P., Zarling, J., and Hu, S.-L., J. Virol. 64, 2653–2659, (1990).
25. Ho, P. C., Mutch, D. A., Winkel, K. D., Saul, A. J., Jones, G. L., Doran, T. J., and Rzepczyk, C. M., Eur. J. Immunol. 20, 477–483 (1990).
26. Hosmalin, A., Nara, P. L., Zweig, M., Lerche, N. W., Cease, K. B., Gard, E. A., Markham, P. D., Putney, S. D., Daniel, M. D., Desrosiers, R. C., and Berzofsky, J. A. J. Immunol. 146, 1667–1673 (1991).
27. Hu, S.-L., Fultz, P., McClure, H., Eichberg, J., Thomas, E., Zarling, J., Singhal, M., Kosowski, S., Swenson, R., Anderson, D., and Todaro, G., Nature 328, 721–723 (1987).
28. Hu, S.-L., Travis, B. M., Garrigues, J., Zarling, J. M., Eichberg, J. W. and Alpers, C. E., In Vaccine 90, eds. Chanock, R. M., Lerner, R. A., Brown, F., and Ginsberg, H., (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.) pp. 231–236 (1990).
29. Hu, S.-L., Kosowski, S., and Dalrymple, J., Nature 320, 535–537, (1986).
30. Hu, S. L., Zarling, J. M., Chinn, J., Travis, B. M., Moran, P. A., Sias, J., Kuller, L., Morton, W. R., Heidecker, G., and Benveniste, R. E., Proc. Natl. Acad. Sci. USA 86, 7213 (1989).
31. Javeherian, K., Langlois, A. J. MCDanal, C., Ross, K. L., Eckler, L. I., Jellib, C. L., Profy, A. T., Rusche, J. R., Bolognesi, D. P., Putney, S. D., and Matthews, T. J. Proc. Natl. Acad. Sci. USA 86, 6768–6772 (1989).
32. Karacostas, V., Nagashima, K., Gonda, M. A., and Moss, B. Proc. Natl. Acad. Sci. USA 86, 8964–8968 (1989).
33. Klasse, P. J., Pipkorn, R., and Blomberg, J., Proc. Natl. Acad. Sci. USA 85, 5225–5229 (1988).
34. Knauf, V. C. and Nester, E. W., Plasmid 8, 45–54 (1982).
35. Kodama, T., Wooley, D. P., Naidu, Y. M., Kestler III, H. W., Daniel, M. D., Li, Y., and Derisiers, R. C. J. Virol. 63, 4709–4714 (1989).
36. Koff, W. C. and Fauci, A. S., AIDS 1, 5125–5129 (1989).
37. Koup, R. A., Sullivan, J. L., Levine, P. H., Brettler, D., Mahr, A., Mazzara, G., McKenzie, S., and Panicali, D. Blood 73, 1909–1919 (1989).
38. Kunkel, T. A., Proc. Natl. Acad. Sci. USA 82, 488–492 (1985).
39. Laemmli, U. K., Nature (London) 227, 680–685 (1970).
40. Lane, J. M., Ruben, F. L., Neff, J. M., and Millar, J. D. New Eng. J. Med. 281, 1201–1208 (1969).
41. LaRosa, G. J., Davide, J. P., Weinhold, K., Waterbury, J. A., Profy, A. T., Lewis, J. A., Langlois, A. J., Dressman, G. R., Boswell, R. N., Shadduck, P., Holley, L. H., Karpus, M., Bolognesi, D. P., Matthews, T. J., Emini, E. A., and Putney, S. D., Science 249, 932–935 (1990).
42. Letvin, N. L., and King, N. W., J. AIDS, 3, 1023–40 (1990)
43. Mandecki, W., Proc. Natl. Acad. Sci. USA 83, 7177–7182 (1986).
44. Maniatis, T., Fritsch, E. F., and Sambrook, J. In Molecular cloning: a laboratory manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) (1982). 45. McMichael, A. J., Gotch, F. M., Noble, G. R. and Beare, P. A. S., New Engl. J. Med. 309, 13–17 (1983)
46. Michel, F., Hoffenbach, A., Langlade-Demoyen, P., Guy, B., Lecocq, J.-P., Wain-Hobson, S., Kieny, M.-P., and Plata, F., Eur. J. Immunology 18, 1917–1927 (1988).
47. Moss, D. J., Rickinson, A. B., and Pope, J. H., Int. J. Cancer 22, 662–668 (1978)
48. Murphy-Corb, M., Martin, L. N., Davison-Fairburn, B., Montelaro, R. C., Miller, M., West, M., Ohkawa, S., Baskin, G. B., Zhang, J.-Y., Putney, S. D., Allison, A. C. and Eppstein, D. A., Science 246, 1293–1297 (1989).
49. Nixon, D. F., Townsend, A. R. M., Elvin, J. G., Rizza, C. R., Gallwey, J., and McMichael, A. J., Nature 326, 484–487 (1988).
50. Panicali, D., and Paoletti, E., Proc. Natl. Acad. Sci. USA 79, 4927–4931 (1982).
51. Perkus, M. E., Piccini, A., Lipinskas, B. R., and Paoletti, E., Science 229, 981–984 (1985).
52. Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Limbach, K., Norton, E. K., and Paoletti, E., Virology 179, 276–286 (1990).
53. Perkus, M. E., Limbach K., and Paoletti, E., J. Virol. 63, 3829–3836 (1989).
54. Piccini, A., Perkus, M. E., and Paoletti, E., Methods in Enzymology 153, 545–563 (1987).
55. Plata, F., Autran, B., Martins, L. P., Wain-Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J.-M., Debre, P., Nature 328, 348–351 (1987).
56. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R. Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., and Wong-Staal, F., Nature 313, 277 (1985).
57. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, Jr., S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo., R. C., and Wong-Staal F., Nature 313, 277–284 (1985).
58. Rautmann, G., Kieny, M.-P., Brandely, R., Dott, K., Girard, M. Montagnier, L., and Lecocg, J.-P. AIDS Research and Human Retroviruses 5, 147–157 (1989).

59. Riviere Y., Tanneau-Salvadori, F., Regnault, A., Lopez, O., Sansonetti, P., Guy, B., Kieny, M.-P., Fournel, J.-J., and Montagnier, L., J. Virol. 63, 2270–2277 (1989).
60. Ruegg, C. L., Monell, C. R., and Strand, M., J. Virol. 63, 3250–3256 (1989a).
61. Ruegg, C. L., Monell, C. R., and Strand, M., J. Virol. 63, 3257–3260 (1989b).
62. Sanchez-Pascador, R., Power, M. D., Barr, P. J., Steimer, K. S., Stampien, M. M., Brown-Shimer, S. L., Gee, W., Renard, A., Randolph, A., Levy, J. A., Dina, D., and Luciw, P. A., Science 227, 484–492 (1985).
63. Sanger, F., Nickeln, S., and Coulson, A. R., Proc. Natl. Acad. Sci. 74, 5463–5467 (1977).
64. Schmaljohn, C. S., Jennings, G. B., Hay, J., Dalrymple, J. M., Virology 155, 633–43 (1986).
65. Schmitt, J. F. C., and Stunnenberg, H. G., J. Virol. 62, 1889–1897 (1988).
66. Shaffermann, A., Lennox, J., Grosfeld, H., Sadoff, J., Redfield, R. R., and Burke, D. S., AIDS Research and Human Retroviruses 5, 33–39 (1989).
67. Shapira, S. K., Chou, J., Richaud, F. V., and Casadaban, M. J., Gene 25, 71–82 (1983).
68. Shioda, T. and H. Shibuta Virology 175, 139–148 (1990).
69. Starcich et al., Cell 45, 637–648 (1986).
70. Tabor, S., and Richardson, C. C., Proc. Natl. Acad. Sci. USA 84, 4767–4771 (1987).
71. Tartaglia, J. and Paoletti, E., In Immunochemistry of viruses, II, eds. van Regenmortel, M. H. V. and Neurath, A. R., (Elsevier Science Publishers B. V., Amsterdam) pp. 125 (1990).
72. Tartaglia, J., Winslow, J., Goebel, S., Johnson, G. P., Taylor, J., and Paoletti, E. J. Gen. Virol. 71, 1517–1524 (1990).
73. Tartaglia, J. Perkus, M. E., Taylor, J. Norton, E. K., Audonnet, J. C., Cox, W. L. Davis, S. W., VanderHoven, J., Meignier, B., Riviere, M., Languet, B. and Paoletti, E., Virology, 188 (1992).
74. Taylor, J., Weinberg, R., Lanquet, B., Desmettre, P., and Paoletti, E., Vaccine 6, 497–503 (1988a).
75. Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R. G., and Paoletti, E., Vaccine 6, 504–508 (1988b).
76. Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E., and Paoletti, E., Virology 187, 321–328 (1992).
77. Vos, J. C., and Stunnenberg, H. G., EMBO J. 7, 3487–3492 (1988).
78. Walker, B. D., Flexner, C., Paradis, T. J., Fuller, T. C., Hirsch, M. S., Schooley, R. T. and Moss, B., Science 240, 64–66 (1988).
79. Walker, B. D., Chakrabarti, S., Moss, B., Paradi, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S., and Schooley, R. T., Nature 328, 345–348 (1987).
80. Walker, B. D., Flexner, C., Birch-Limberger, K., Fisher, L., Paradis, T. J., Aldovini, A., Young, R., Moss, B., and Schooley, R. T., Proc. Natl. Acad. Sci. 86, 9514–9519 (1989).
81. Walker, B. D. and Plata, F., AIDS 4, 177–184 (1990)
82. Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, G., Carne, C. A., Weller, I. V., and Tedder, R. S., Nature 316, 69–72 (1985).
83. Yuen, L., and Moss, B., Proc. Natl. Acad. Sci. USA 84, 6417–6421 (1987).
84. Zagury, D., Bernard, J., Cheynier, R., Desportes, I., Leonard, R., Fouchard, I., Reveil, B., Ittele, D., Lurhuma, Z., Mbayo, K., Wane, J., Salaun, J.-J., Goussard, B., Dechazal, L., Burny, A., Nara, P. and Gallo, R. C., Nature 332, 728–731 (1988).
85. Zarling, J. M., Morton, W., Moran, P. A., McClure, J., Kosowski, S. G., and Hu, S.-L., Nature 323, 344–346 (1986).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 205

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAATTAACTA GCTACCCGGG 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATTCCCGGG TAGCTAGTTA ATTACATG 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCTTCCCGG GTAAGTAATA CGTCAAGGAG AAAACGAAAC GATCTGTAGT TAGCGGCCGC 60

CTAATTAACT AAT 73

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATTAGTTAAT TAGGCGGCCG CTAACTACAG ATCGTTTCGT TTTCTCCTTG ACGTATTACT 60

TACCCGGGA 69

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTAGTTAATT AGGCGGCCGC 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGATTACTAT GAAGGATCCG TT 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AACGGATCCT TCATAGTAAT 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGATTACTAG ATCTGAGCTC CCCGGGCTCG AGGGATCCGT T 41

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AACGGATCCC TCGAGCCCGG GGAGCTCAGA TCTAGTAAT 39

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATCCGAATT CTAGCT 16

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTAGAATT CG 12

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATGAGTAAC TTAACTCTTT TGTTAATTAA AAGTATATTC AAAAAATAAG TTATATAAAT 60

AGATCTGAAT TCGTT 75

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AACGAATTCA GATCTATTTA TATAACTTAT TTTTTGAATA TACTTTTAAT TAACAAAAGA 60

GTTAAGTTAC TCA 73

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAAATGGGCG TGGATTGTTA ACTTTATATA ACTTATTTTT TGAATATAC 49

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ACACGAATGA TTTTCTAAAG TATTTGGAAA GTTTTATAGG TAGTTGATAG AACAAAATAC 60

ATAATTT 67

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCTATCAACT ACCTATAAAA CTTTCCAAAT ACTTTAGAAA ATCATTCGTG T 51

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGTAAAAATA AATCACTTTT TATACTAAGA TCTCCCGGGC TGCAGC 46

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGCCGCTGCA GCCCGGGAGA TCTTAGTATA AAAAGTGATT TATTTTTACA AAATTATGTA 60

TTTTGT 66

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTTCTGTATA TTTGCACCAA TTTAGATCTT ACTCAAAATA TGTAACAATA 50

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGTCATTTAA CACTATACTC ATATTAATAA AAATAATATT TATT 44

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GATCCTGAGT ACTTTGTAAT ATAATGATAT ATATTTTCAC TTTATCTCAT TTGAGAATAA 60

AAAGATCTTA GG 72

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AATTCCTAAG ATCTTTTTAT TCTCAAATGA GATAAAGTGA AAATATATAT CATTATATTA 60

CAAAGTACTC AG 72

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATCCAGATC TCCCGGGAAA AAAATTATTT AACTTTTCAT TAATAGGGAT TTGACGTATG 60

TAGCGTACTA GG 72

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AATTCCTAGT ACGCATCATA CGTCAAATCC CTATTAATGA AAAGTTAAAT AATTTTTTTC 60

CCGGGAGATC TG 72

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATGGTAGAAA TTAATTGTAC 20

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCATCGAAT TCAAGCTTAT TATTTTGCTC TACTAATGTT AC 42

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATGAATGTGA CAGAAAATTT TAACATGTGG AAAAATGTAG AAATTAATTG TACAAGACCC 60

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGGTCTTGTA CAATTAATTT CTACATTTTT CCACATGTTA AAATTTTCTG TCACATTCAT 60

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGTAATGTGA CAGAAAATTT TAAC 24

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGGCAAGCTT TCAAAAAAAT ATAAATGATT C 31

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TTTATATTGT AATTATATAT TTTC 24

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GTTTTAATTG TGGAGGGGAA TTCTTCTACT GTAATTC 37

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ATCATCTCTA GAATAAAAAT TATAGCAAAA TCCTTTC 37

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TGCTACTCCT AATGGTTC 18

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CATATGCTTT AGCATCTGAT G 21

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATGAAAGAGC AGAAGACAGT G 21

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ATCATCGGTA CCGATTCTTT ATTCTATAC 29

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TACGATACAA ACTTAACGG 19

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GAATTACAGT AGAAGAATTC CCCTCCACAA TTAAAAC 37

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CAATAGATAA TGATACTAC 19

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GTATTATATC AAGTTTATAT AATAATGCAT ATTC 34

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTTGATGATC TGTAGTGC 18

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ATCATCTCTA GAATAAAAAT TATGGTTCAA TTTTTACTAC TTTTATATTA TATATTTC 58

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CAATAATCTT TAAGCAAATC CTC 23

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AGAGGGGAAT TCTTCTACTG CAATACA 27

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Leu Gln Ala Arg Val Leu Ala Val Glu Arg Tyr Leu Arg Asp Gln Gln
1 5 10 15

Leu

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTGGAAAGGC TTTTGGCATG CCACGCGTC 29

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

ACAGTCTGGG GCATCAAGCA GCTAGGGATT TGGGGTTGCT CT 42

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CCGTTAAGTT TGTATCGTAA TGAAAGTGAA GGGGACCAGG 40

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:50:

ATGAGTGGTA AAATTCAGCT GCTTGTTGCC TTTCTGCTAA CTAGTGCTTG CTTA 54

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TAAGCAAGCA CTAGTTAGCA GAAAGGCAAC AAGCAGCTGA ATTTTACCAC TCAT 54

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:52:

ATCATCAAGC TTGATTCTTT ATTCTATAC 29

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CAGCTGAATT TTACCACTCA TTACGATACA AACTTAACG 39

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TAAGCAAGCA CTAGTTAG 18

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CCGCCTCTTG ACCAGAC 17

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:56:

ATCATCTCTA GAATAAAAAT TACAGGAGGG CAATTTCTG 39

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ATCATCTCTA GAATAAAAAT TATCTCTTAT GTCTCCCTGG 40

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:58:

AATTAACTTT ACAGCACC 18

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CGATATCCGT TAAGTTTGTA TCGTAATGGG ATGTCTTGGG AATC 44

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CAAGGCTTTA TTGAGGTCTC 20

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

CCTGGCCTTG GCAGATAG 18

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:62:

ATCATCGAAT TCAAAAATAT TACAAAGAGC GTGAGCTCAA GTCCTTGCCT AATCCTCC 58

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CCCCCCAAGC TTTTTTATTC TATACTT 27

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CAAGGCTTTA TTGAGGTCTC 20

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CAGTTGGTAC CACTGGTATT TTATTTCAG 29

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TATCTGAATT CCTGCAGCCC GGGTTTTTAT AGCTAATTAG TCAAATGTGA GTTAATATTA 60

G 61

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TCGCTGAATT CGATATCAAG CTTATCGATT TTTATGACTA GTTAATCAAA TAAAAAGCAT 60

ACAAGC 66

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TTATCGAGCT CTGTAACATC AGTATCTAAC 30

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TCGAGTGAGA TAAAGTGAAA ATATATATCA TTATATTACA AGTACAATTA TTTAGGTTTA 60

ATCATGGGCG 70

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCCATGATTA AACCTAAATA ATTGTACTTT GTAATATAAT GCTATATATT TTCACTTTAT 60

CTCAC 65

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:71:

AATCAGAGAG CAGGCT 16

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:72:

TTGGATCCCT ATGCCACCTC TCT 23

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:73:

AGACCAACAG CACCATCTAG CGGCAGAGGA GGAAATTACT AATTTTTATT CTAGAG 56

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GATCCTCTAG AATAAAAATT AGTAATTTCC TCCTCTGCCG CTAGATGGTG CTGTTGGT 58

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TAGACAAAAT TGAAAATATA TAATTACAAT ATAAAATGCC AGTACAACAA ATAGGTGGTA 60

ACTATGTCCA CCTGCCATT 79

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GCTTAATGGC AGGTGGACAT AGTTACCACC TATTTGTTGT ACTGGCATTT TATATTGTAA 60

TTATATATTT TCAATTTTGT 80

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:77:

TGGATGTACA GACAAC 16

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:78:

AAGGATCCGA ATTCTTACAT TAATCTAGCC TTC 33

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Cys Asn Thr Arg Lys Arg Ile Arg Ile Gln Arg Gly Pro Gly Arg Ala
1 5 10 15

Phe Val Thr Gly Lys
20

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Cys Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr
1 5 10 15

Thr Thr Lys Asn
20

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Cys Asn Thr Arg Lys Ser Ile Tyr Ile Gly Pro Gly Arg Ala Phe His
1 5 10 15

Thr Thr Gly Arg
20

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GGGTTATTAA TGATCTGTAG 20

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:83:

ATCATCGAGC TCTGTTCCTT GGGTTCTTAG 30

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:84:

ATCATCTCTA GAATAAAAAT TATAGCAAAG CCCTTTCCAA GCC 43

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:85:

ATCATCGAGC TCCTATCGCT GCTC 24

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:86:

AGCTTCTTTA TTCTATACTT AAAAAGTGAA AATAAATACA AAGGTTCTTG AGGGT 55

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TGTGTTAAAT TGAAAGCGAG AAATAATCAT AAATTATTTC ATTATCGCGA TATCCGTTAA 60

GTTTGTATCG TAC 73

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:88:

TTATTAGTAT TTAATAAAGT AATAGCGCTA TAGGCAATTC AAACATAGCA TGAGCT 56

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:89:

AGAAATAAGA TATGAATTTT TCACTTTTAT TTATGTTTCC AAGAACTCCC AACACAATTT 60

AACTTTCGCT CT 72

(2) INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:90:

ATCATCGAAT TCTGAATGTT AAATGTTATA CTTTG 35

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

GGGGGTACCT TTGAGAGTAC CACTTCAG 28

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

ATCATCCTGC AGGTATTCTA AACTAGGAAT AGATG 35

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ATCATCCTGC AGGTATTCTA AACTAGGAAT AGATG 35

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

AATACGACTC ACTATAG 17

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

ATCATCTCTA GAATAAAAAT TATCTTTTTT CTCTCTGCAC CACTC 45

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GAAATAATAA AACAATAATC 20

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:97:

GCTCCTATTC CCACTGCAGT TTTTTCTCTC TGCAC 35

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:98:

GTCCTGCAGG ATGGAAAAGA ATGCCCCAAG C 31

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGGGAGGCA AACTACCAAG G 21

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:100:

ATCATCTCTA GAATAAAAAT TAGAGTTTCA AAGGC 35

(2) INFORMATION FOR SEQ ID NO:101:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:101:

CGCCAGCATG CAGAAGCAGC 20

(2) INFORMATION FOR SEQ ID NO:102:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:102:

ATCATCTCTA GAATAAAAAT TATAGGAAAG CCCTTTCCAA GCC 43

(2) INFORMATION FOR SEQ ID NO:103:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:103:

GTGCAGAGAA AAAACTGCAG TGGGAATAGG AGC 33

(2) INFORMATION FOR SEQ ID NO:104:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:104:

ATCATCTCTA GAATAAAAAT TACAAACTTG CCCATTTATC CAATTCC 47

(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:105:

ATCATCTCTA GAATAAAAAT TACAAACTTG CCCATTTATC TAATTCC 47

(2) INFORMATION FOR SEQ ID NO:106:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:106:

GCCTCCTACT ATCATTATGA ATAATCTTTT TCTCTCTG 38

(2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:107:

TTATTCATAA TGATAGTAGG AGGCTTGGTA GGTTTAAGAA TAGTTTTTGC TGTACTCTCT 60

GTAGTGAATA GAGTTAGGCA GGGATAA 87

(2) INFORMATION FOR SEQ ID NO:108:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:108:

TTATCCCTGC CTAACTCTAT TCACTACAGA GAGTACAGCA AAAACTATTC TTAAACCTAC 60

CAAGCCTCCT ACTATCATTA TGAATAA 87

(2) INFORMATION FOR SEQ ID NO:109:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:109:

ATCATCTCTA GAATAAAAAT TATCCCTGCC TAACTCTATT CAC 43

(2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:110:

GTACGTGACT AATTAGCTAT AAAAAGGATC CGGTACCCTC GAGTCTAGAA TCGATCCCGG 60

GTTTTTATGA CTAGTTAATC AC 82

(2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GGCCGTGATT AACTAGTCAT AAAAACCCGG GATCGATTCT AGACTCGAGG GTACCGGATC 60

CTTTTTATAG CTAATTAGTC AC 82

(2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:112:

GATCTTAATT AATTAGTCAT CAGGCAGGGC GAGAACGAGA CTATCTGCTC GTTAATTAAT 60

TAGGTCGACG 70

(2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:113:

GATCCGTCGA CCTAATTAAT TAACGAGCAC ATAGTCTCGT TCTCGCCCTG CCTGATGACT 60

AATTAATTAA 70

(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:114:

GGTCGACGGA TCCT 14

(2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:115:

GATCAGGATC CGTCGACCTG CA 22

(2) INFORMATION FOR SEQ ID NO:116:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:116:

TGTGGCAAAG AAGGGC 16

(2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:117:

TTGGATCCTT ATTGTGACGA GGGGTC 26

(2) INFORMATION FOR SEQ ID NO:118:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:118:

GATCTTGAGA TAAAGTGAAA ATATATATCA TTATATTACA AAGTACAATT ATTTAGGTTT 60

AATCATGGGT GCGAGAGCGT CAGTATTAAG CGGGGGAGAA TTAGAT 106

(2) INFORMATION FOR SEQ ID NO:119:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:119:

CGATCTAATT CTCCCCCGCT TAATACTGAC GCTCTCGCAC CCATGATTAA ACCTAAATAA 60

TTGTACTTTG TAATATAATG ATATATATTT TCACTTTATC TCAA 104

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:120:

CTGACACAGG ACACAGCAAT CAGGTCAGCC AAAATTACTA ATTTTTATCT CGAGGTCGAC 60

AGGACCCG 68

(2) INFORMATION FOR SEQ ID NO:121:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

GATCCGGGTC CTGTCGACCT CGAGATAAAA ATTAGTAATT TTGGCTGACC TGATTGCTGT 60

GTCCTGTGTC AG 72

(2) INFORMATION FOR SEQ ID NO:122:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

AAGAAAATTA TAGGAC 16

(2) INFORMATION FOR SEQ ID NO:123:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

TTGGATCCCT AATCCTCATC CTGT 24

(2) INFORMATION FOR SEQ ID NO:124:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

AAAGTCGACC CATATCACCT AGAAC 25

(2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

TTTGGATCCT TACAAAACTC TTGCCTTAT 29

(2) INFORMATION FOR SEQ ID NO:126:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 105 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

-continued

TCGAGCAAAA TTGAAAATAT ATAATTACAA TATAAAATGC CTATAGTGCA GAACATCCAG 60

GGGCAAATGG TACATCAGGC CATATCACCT AGAACTTTAA ATGCA 105

(2) INFORMATION FOR SEQ ID NO:127:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:127:

TTTAAAGTTC TAGGTGATAT GGCCTGATGT ACCATTTGCC CCTGGATGTT CTGCACTATA 60

GGCATTTTAT ATTGTAATTA TATATTTTCA ATTTTGC 97

(2) INFORMATION FOR SEQ ID NO:128:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:128:

GCCTCCTACT ATCATTATGA ATAAACTGAT GGGAGGGGCA TAC 43

(2) INFORMATION FOR SEQ ID NO:129:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:129:

GGCCGCAAC 9

(2) INFORMATION FOR SEQ ID NO:130:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:130:

TCGAGTTGC 9

(2) INFORMATION FOR SEQ ID NO:131:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:131:

GGCCAAAC 8

(2) INFORMATION FOR SEQ ID NO:132:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:132:

TCGAGTTT 8

(2) INFORMATION FOR SEQ ID NO:133:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:133:

CCCCCCAAGC TTACATCATG CAGTGGTTAA AC 32

(2) INFORMATION FOR SEQ ID NO:134:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:134:

GATTAAACCT AAATAATTGT 20

(2) INFORMATION FOR SEQ ID NO:135:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:135:

ACAATTATTT AGGTTAACTG CA 22

(2) INFORMATION FOR SEQ ID NO:136:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:136:

GTTAACCTAA ATAATTGT 18

(2) INFORMATION FOR SEQ ID NO:137:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:137:

TAATCATGAA ACAAATTATT AATATGTGGC AAGAAGAGGA AAAGCTATGT ACGCTTGACT 60

AGTTAATCAC TCGAG 75

(2) INFORMATION FOR SEQ ID NO:138:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:138:

GATCCTCGAG TGATTAACTA GTCAAGCGTA CATAGCTTTT CCTACTTCTT GCCACATATT 60

AATAATTTGT TTCATGATTA 80

(2) INFORMATION FOR SEQ ID NO:139:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:139:

ATCCGTTAAG TTTGTATCGT AATGCACGAA GATATTATTT CTTTGTGGGA TCAATCTTTA 60

AAATGACTAG TTAATCAG 78

(2) INFORMATION FOR SEQ ID NO:140:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:140:

GATCCTGATT AACTAGTCAT TTTAAAGATT GATCCCACAA AGAAATAATA TCTTCGTGCA 60

TTACGATACA AACTTAACGG AT 82

(2) INFORMATION FOR SEQ ID NO:141:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:141:

AATTAATTAG CTGCAGCCCC GGGTCAAAAA AATATAAATG 40

(2) INFORMATION FOR SEQ ID NO:142:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:142:

CCTTGTACTA CTTCAATTAC TCTATCCATT TTATATTGTA ATTATATATT TTC 53

(2) INFORMATION FOR SEQ ID NO:143:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:143:

TCAAAAAAAT ATAAATGATT CACCATCTGA TAGAAAAAAA ATTTATTGGG AAGAATATGA 60

TAATATTTTG GGATTTCAAA ATTGAAAATA TATAATTACA ATATAAA 107

(2) INFORMATION FOR SEQ ID NO:144:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:144:

ATGGATAGAG TAATTGAAGT AGTACAAGGA GCTTATAGAG CTATTAGATG ACTAGTTAAT 60

CACTCGAGGA TCC 73

(2) INFORMATION FOR SEQ ID NO:145:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GGATCCTCGA GTGATTAACT AGTCATCTAA TAGCTCTATA AGCTCCTTGT ACTACTTCAA 60

TTACTCTATC CAT 73

(2) INFORMATION FOR SEQ ID NO:146:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:146:

ATCATCGGAT CCTCGAGTGA TTAAACTAGT CATCTAATAG CTC 43

(2) INFORMATION FOR SEQ ID NO:147:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:147:

TTAATCAGGA TCCTTAATTA ATTAGTTATT AGACAAGGTG AAAACGAAAC TATTTGTAGC 60

TTAATTAATT AGCTGCAGCC CGGG 84

(2) INFORMATION FOR SEQ ID NO:148:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:148:

CCCGGGCTGC AGCTAATTAA TTAAGCTACA AATAGTTTCG TTTTCACCTT GTCTAATAAC 60

TAATTAATTA AGGATCCTGA TTAA 84

(2) INFORMATION FOR SEQ ID NO:149:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:149:

TTAATCAGGA TCCTTAATTA ATTAGTTATT AGAC 34

(2) INFORMATION FOR SEQ ID NO:150:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:150:

ATCATCGGAT CCTCGAGTGA TTAACTAGTC ATCTAATAGC TC 42

(2) INFORMATION FOR SEQ ID NO:151:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:151:

AATTGCGGCC GC 12

(2) INFORMATION FOR SEQ ID NO:152:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:152:

AGAAAAATCA GTTAGCTAAG ATCTCCCGGG CTCGAGGGTA CCGGATCCTG ATTAGTTAAT 60

TTTTGT 66

(2) INFORMATION FOR SEQ ID NO:153:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GATCACAAAA ATTAACTAAT CAGGATCCGG TACCCTCGAG CCCGGGAGAT CTTAGCTAAC 60

TGATTTTTCT 70

(2) INFORMATION FOR SEQ ID NO:154:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:154:

Met Lys Glu Gln Lys Thr Val Ala Met Arg Val Lys Glu Lys Tyr Gln
1 5 10 15

His Leu Trp Arg Trp Gly Trp Arg Trp Gly Thr Met Leu Leu Gly Met
20 25 30

Leu Met Ile Cys Ser Ala Thr Glu Lys Leu Trp Val Thr Val Tyr Tyr
35 40 45

Gly Val Pro

50

(2) INFORMATION FOR SEQ ID NO:155:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:155:

Pro Phe Arg Lys Gln Ile Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Phe Arg Lys His Glu Asp Ile Ile Ser Leu Trp
20 25 30

Asp Gln Ser Leu Lys Pro Pro Phe Arg Lys Asp Arg Val Ile Glu Val
35 40 45

Val Gln Gly Ala Tyr Arg Ala Ile Arg Pro Pro Phe Arg Lys
50 55 60

(2) INFORMATION FOR SEQ ID NO:156:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:156:

Leu Phe Ile Met Ile Val Gly Gly Leu Val Gly Leu Arg Ile Val Phe
1 5 10 15

Ala Val Leu Ser Val Val Asn Arg Val Arg Gln Gly
20 25

(2) INFORMATION FOR SEQ ID NO:157:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:157:

ACTACTAAGC TTCTTTATTC TATACTTAAA AAGTG 35

(2) INFORMATION FOR SEQ ID NO:158:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:158:

CATATTAATT TGTTTTCTAA AAGGAGGTAC CCCATAATAG ACTGTG 46

(2) INFORMATION FOR SEQ ID NO:159:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 94 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:159:

GCTCCTCCTT TTAGAAAACA CGAAGATATT ATTTCTTTGT GGGATCAATC TTTAAAACCT 60

CCTTTTAGAA AAGATAGAGT AATTGAAGTA GTAC 94

(2) INFORMATION FOR SEQ ID NO:160:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 94 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:160:

GTACTACTTC AATTACTCTA TCTTTTCTAA AAGGAGGTTT TAAAGATTGA TCCCACAAAG 60

AAATAATATC TTCGTGTTTT CTAAAAGGAG GAGC 94

(2) INFORMATION FOR SEQ ID NO:161:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:161:

AAACAAATTA TTAATATGTG GCAAGAAGTA GGAAAAGCTA TGTACGCTCC TCCTTTTAGA 60

AAACACGAAG 70

(2) INFORMATION FOR SEQ ID NO:162:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:162:

ACTACTTCTA GATTATCTAA TAGCTCTATA AGCTCCTTGT ACTACTTCAA TTACTC 56

(2) INFORMATION FOR SEQ ID NO:163:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:163:

TACTATCATT ATGAATAATT TTCTAAAAGG AGGTCTAATA GCTCTATAAG CTCCTTGTAC 60

TACTTCAATT ACTC 74

(2) INFORMATION FOR SEQ ID NO:164:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:164:

ATCATCGGAT CCAAGCTTAC ATCATGCAGT GG 32

(2) INFORMATION FOR SEQ ID NO:165:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:165:

CGTTTTGACC ATTTGCCACC CATGATTAAA CCTAAATAAT TGTACTTTG 49

(2) INFORMATION FOR SEQ ID NO:166:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:166:

AGTACAATTA TTTAGGTTTA ATCATGGGTG GCAAATGGTC AAAACG 46

(2) INFORMATION FOR SEQ ID NO:167:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:167:

ATCATCGGAT CCTAACACTT CTCTCTCCGG 30

(2) INFORMATION FOR SEQ ID NO:168:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:168:

ATCATCGGAT CCTAACACTT CTCTCTCCGG GTCATCCATC CATGCTGGCT CATAG 55

(2) INFORMATION FOR SEQ ID NO:169:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:169:

AATTAACCCG GGATCCAAGC TTCTAGCTAG CTAATTTTTA TAGCGGCCGC TATAATCGTT 60

AACTTATTAG 70

(2) INFORMATION FOR SEQ ID NO:170:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:170:

GCTAGAAATC TCTTAGTTTT TATAGTTG 28

(2) INFORMATION FOR SEQ ID NO:171:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:171:

GTTACATATG TACAGAATCT GATCATAG 28

(2) INFORMATION FOR SEQ ID NO:172:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:172:

CTAGCTAGAA GCTTGGATCC CGGGTTAATT AATTAATAAA AAGCGGCCGC GTTAAAGTAG 60

AAAAATG 67

(2) INFORMATION FOR SEQ ID NO:173:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:173:

CGCCCATGAT TAAACCTAAA TAATTGTACT TTGTAATATA ATGATATATA TTTTCACTTT 60

ATCTCAC 67

(2) INFORMATION FOR SEQ ID NO:174:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:174:

ATGGCAGTTC ATTGCAT 17

(2) INFORMATION FOR SEQ ID NO:175:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:175:

TTCCCGGGAG ATCTCTATGC CATTTCTCCA T 31

(2) INFORMATION FOR SEQ ID NO:176:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:176:

GGTTG 5

(2) INFORMATION FOR SEQ ID NO:177:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:177:

AATTCAACCG C 11

(2) INFORMATION FOR SEQ ID NO:178:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:178:

CTAGCTAAGT TAAGGCAGGG GTATAGGCCA GTGTTCTCTT CCCCACCCTC TTATTTCCAG 60

CAGACTCATA CCCAACAG 78

(2) INFORMATION FOR SEQ ID NO:179:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:179:

GTCCTGTTGG GTATGAGTCT GCTGGAAATA AGAGGGTGGG GAAGAGAACA CTGGCCTATA 60

CCCCTGCCTT AACTTAG 77

(2) INFORMATION FOR SEQ ID NO:180:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:180:

AAAGGATCCC CCGGGTTAAA AATTTAAAGT GCAACC 36

(2) INFORMATION FOR SEQ ID NO:181:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:181:

CGATAAACCG C 11

(2) INFORMATION FOR SEQ ID NO:182:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:182:

GGTTTAT 7

(2) INFORMATION FOR SEQ ID NO:183:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:183:

GGGAAAG 7

(2) INFORMATION FOR SEQ ID NO:184:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:184:

GATCCTTTCC C 11

(2) INFORMATION FOR SEQ ID NO:185:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:185:

CTAGAAAACC GC 12

(2) INFORMATION FOR SEQ ID NO:186:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:186:

GGTTTT 6

(2) INFORMATION FOR SEQ ID NO:187:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:187:

CAGAAGTAGC ATATATGT 18

(2) INFORMATION FOR SEQ ID NO:188:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:188:

GCCTCCTACT ATCATTATGA ATAATCTCTT ATGTCTCCCT GGAGC 45

(2) INFORMATION FOR SEQ ID NO:189:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:189:

```
Met Lys Glu Gln Lys Thr Val Ala Met Arg Val Lys Glu Lys Tyr Gln
1               5                   10                  15

His Leu Trp Arg Trp Gly Trp Arg Trp Gly Thr Met Leu Leu Gly Met
            20                  25                  30

Leu Met Ile Cys Ser Ala Thr Glu Lys Leu Trp Val Thr Val Tyr Tyr
        35                  40                  45

Gly Val Pro Pro Phe Arg Lys
    50                  55
```

(2) INFORMATION FOR SEQ ID NO:190:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:190:

```
Pro Phe Arg Lys Gly Pro Lys Glu Pro Phe Arg Asp Tyr Val Asp Arg
1               5                   10                  15

Phe Tyr Lys Pro Pro Phe Arg Lys Val Gln Ile Asn Cys Thr Arg Pro
            20                  25                  30

Asn Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe
        35                  40                  45

Tyr Thr Thr Lys Asn Ile Ile Gly Thr Ile Arg Gln Ala His Cys Asn
    50                  55                  60

Ile Ser Arg Ala Lys Pro Pro Phe Arg Lys Gln Ile Ile Asn Met Trp
65                  70                  75                  80

Gln Glu Val Glu Lys Ala Met Tyr Ala
                85
```

(2) INFORMATION FOR SEQ ID NO:191:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:191:

```
AGGTCCCTTC CTGAATGGAG GTACCCCATA ATAGACTG                              38
```

(2) INFORMATION FOR SEQ ID NO:192:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:192:

```
CCATTCAGGA AGGGACCTAA AGAACCTTTT AGAGATTATG TAGATAGATT TTATAAACCA      60

CCTTTTAGAA AA                                                          72
```

(2) INFORMATION FOR SEQ ID NO:193:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:193:

TTTTCTAAAA GGTGGTTTAT AAAATCTATC TACATAATCT CTAAAAGGTT CTTTAGGTCC 60

CTTCCTGAAT GG 72

(2) INFORMATION FOR SEQ ID NO:194:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:194:

GTACAATTAA TTTGTACTTT TCTAAAAGGT GGTTTATAAA ATC 43

(2) INFORMATION FOR SEQ ID NO:195:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:195:

GGTGGTTTAT AAAATCTATC TACATAATCT CTAAAAGGTT CTTTAGGTCC CATTACGATA 60

CAAACTTAAC GG 72

(2) INFORMATION FOR SEQ ID NO:196:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:196:

AAACCACCTT TTAGAAAAGT ACAAATTAAT TGTAC 35

(2) INFORMATION FOR SEQ ID NO:197:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:197:

CTGCTTACGG AACGGTGGTT TTGCTCTACT AATGTTACAA TG 42

(2) INFORMATION FOR SEQ ID NO:198:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:198:

CCACCGTTCC GTAAGCAGAT AATAAACATG TGGCAAGAAG TAGAAAAAGC TATGTATGCT 60

TAA 63

(2) INFORMATION FOR SEQ ID NO:199:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:199:

TTAAGCATAC ATAGCTTTTT CTACTTCTTG CCACATGTTT ATTATCTGCT TACGGAACGG 60

TGG 63

(2) INFORMATION FOR SEQ ID NO:200:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:200:

TCATCAAAGC TTCTCGAGAA AAATTAAGCA TACATAGCTT TTTC 44

(2) INFORMATION FOR SEQ ID NO:201:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:201:

ATCATCGAGC TCGCGGCCGC CTATCAAAAG TCTTAATGAG TT 42

(2) INFORMATION FOR SEQ ID NO:202:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:202:

GAATTCCTCG AGCTGCAGCC CGGGTTTTTA TAGCTAATTA GTCATTTTTT CGTAAGTAAG 60

TATTTTTATT TAA 73

(2) INFORMATION FOR SEQ ID NO:203:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:203:

CCCGGGCTGC AGCTCGAGGA ATTCTTTTTA TTGATTAACT AGTCAAATGA GTATATATAA 60

TTGAAAAAGT AA 72

(2) INFORMATION FOR SEQ ID NO:204:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:204:

GATGATGGTA CCTTCATAAA TACAAGTTTG ATTAAACTTA AGTTG 45

(2) INFORMATION FOR SEQ ID NO:205:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:205:

TTAACGGATA TCGCGATAAT G 21

What is claimed is:

1. A recombinant attenuated ALVAC canarypox virus comprising an exogenous DNA segment encoding a lentivirus gene product.

2. The recombinant virus of claim 1 wherein the exogenous DNA segment encodes a human immunodeficiency virus or simian immunodeficiency virus gene product.

3. The recombinant virus of claim 2 wherein the exogenous DNA segment encodes a human immunodeficiency virus gene product.

4. The recombinant virus of claim 2 wherein the exogenous DNA segment encodes a simian immunodeficiency virus gene product.

5. The recombinant virus of claim 1 wherein said exogenous DNA segment further encodes a human T-helper lymphocyte epitope derived from tetanus toxoid fragment C.

6. The recombinant virus of claim 5 wherein said exogenous DNA segment encodes a human immunodeficiency virus or simian immunodeficiency virus gene product.

7. The recombinant virus of claim 6 wherein said exogenous DNA segment encodes a human immunodeficiency virus gene product.

8. The recombinant virus of claim 6 wherein said exogenous DNA segment encodes a simian immunodeficiency virus gene product.

9. A recombinant attenuated ALVAC canarypox virus selected from the group consisting of vCP95, vCP112, vCP60, vCP61, vCP125, vCP124, vCP126, vCP144, vCP120, vCP138, vCP117, vCP130, vCP152, vCP155, vCP156, vCP146, vCP148, vCP154, vCP168, vCP153, and vCP172.

10. The recombinant virus of claim 1 wherein the gene product is selected from the group consisting of: gp160; gp120; Gag and Pol; Gag, Pol and gp120; Gag, Pol and gp160; Gag, Pol and truncated Env; non-cleavable gp160; gp120 anchored with transmembrane; Gag, Pol, and gp120 anchored with transmembrane; signal domain of Env and p24 fused to T1 and V3 loop of Env; p24 fused to T1 and V3 loop of Env; Nef; V3 loop fused to 88 epitope; T1, T2, and TH4.1 epitopes; Env signal domain, T1, T2, and TH4.1 epitopes; and, Gag, Pol protease and gp120 anchored with a transmembrane sequence.

11. An immunogenic composition comprising a recombinant attenuated canarypox virus as claimed in any one of claims 1 to 10 and an adjuvant.

12. A method for expressing a lentiviral gene product comprising infecting a suitable host cell with a recombinant attenuated canarypox virus as claimed in any one of claims 1 to 10.

13. A method of inducing an immunological response to a lentivirus gene product comprising administering a recombinant attenuated canarypox virus as claimed in any one of claims 1 to 10.

14. A method of inducing an immunological response to a lentivirus gene product comprising administering an immunogenic composition as claimed in claim 11.

* * * * *

US005866136A

United States Patent [19]

Ramshaw et al.

[11] Patent Number: 5,866,136

[45] Date of Patent: Feb. 2, 1999

[54] RECOMBINANT VACCINE

[75] Inventors: **Ian Allister Ramshaw**, Australian Capital Territory; **David Bernard Boyle**, Victoria; **Barbara Elizabeth Howieson Coupar**, Victoria; **Marion Elizabeth Andrew**, Victoria, all of Australia

[73] Assignees: **Commonwealth Scientific and Industrial Organisation; The Australian National University**, both of Australian Capital Territory, Australia

[21] Appl. No.: **611,112**

[22] Filed: **Nov. 9, 1990**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 498,420, Mar. 26, 1990, abandoned, which is a continuation of Ser. No. 203,060, filed as PCT/AU87/00246, Jul. 31, 1987, abandoned.

[30] **Foreign Application Priority Data**

Aug. 1, 1986 [AU] Australia PH07212/86

[51] **Int. Cl.**^{6} **C12N 15/64**; C12N 15/86; A61K 39/12

[52] **U.S. Cl.** **424/199.1**; 424/93.2; 435/172.3; 435/320.1

[58] **Field of Search** 424/89, 93.2, 199.1; 435/172.3, 236, 320.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,631,191 12/1986 Dale et al. 424/186.1

FOREIGN PATENT DOCUMENTS

0 138 133 4/1985 European Pat. Off. .
0 176 170 4/1986 European Pat. Off. .
0 181 117 5/1986 European Pat. Off. .

OTHER PUBLICATIONS

J.-P. Van Eendenburg et al., AIDS Res. and Hum. Retrovir. 5(1):41–50 (1989).

Leong et al., "Generation of Enhanced Immune Responses by Consecutive Immunzation with DNA and Recombinant Fowl Pox Vectors," In: *Vaccines 95*, Cold Spring Harbor Press, 1995, pp. 327–331.

Dinarello, *Adv. Immunopharmacol.*, 3, Proc. Int. Conf. 3rd., 1985 (publ. 1986), pp. 223–230.

Bektemirov et al., *Chemical Abstracts*, 92, No. 19, May 12, 1980, p. 446, Abstract No. 162033q.

Yokota et al., *Proc. Natl. Acad. Sci. USA*, 83, No. 16, Aug., 1986, pp. 5894–5898.

Hu et al., *Nature*, 320, Apr. 1986, pp. 537–540.

Cremer, *The Yearbook of Agriculture*, U.S. Dept. of Agriculture, 1986, pp. 104–104.

B.D. Davis et al, Eds. *Microbiology*, Hagerstown: Harper and Row; 1980, p. 294.

S.L. Hu et al. (1983) Nature 302:490–495.

Animal Pharm. (1986) Mar. 14, vol. 100, p. 19. (Abstract).

Primary Examiner—Johnny F. Railey, II
Attorney, Agent, or Firm—Bacon & Thomas

[57] **ABSTRACT**

A recombinant vaccine comprises a vaccine vector which incorporates a first nucleotide sequence capable of being expressed as all or a part of an antigenic polypeptide, together with a second nucleotide sequence capable of being expressed as all or a part of a lymphokine effective in enhancing the immune response to the antigenic polypeptide. The vaccine vectors include poxvirus, herpes virus or adenovirus, and the lymphokine may be an interleukin, tumour necrosis factor or gamma-interferon. The vaccine vector may express an antigenic polypeptide which is foreign to the host vector.

3 Claims, 17 Drawing Sheets

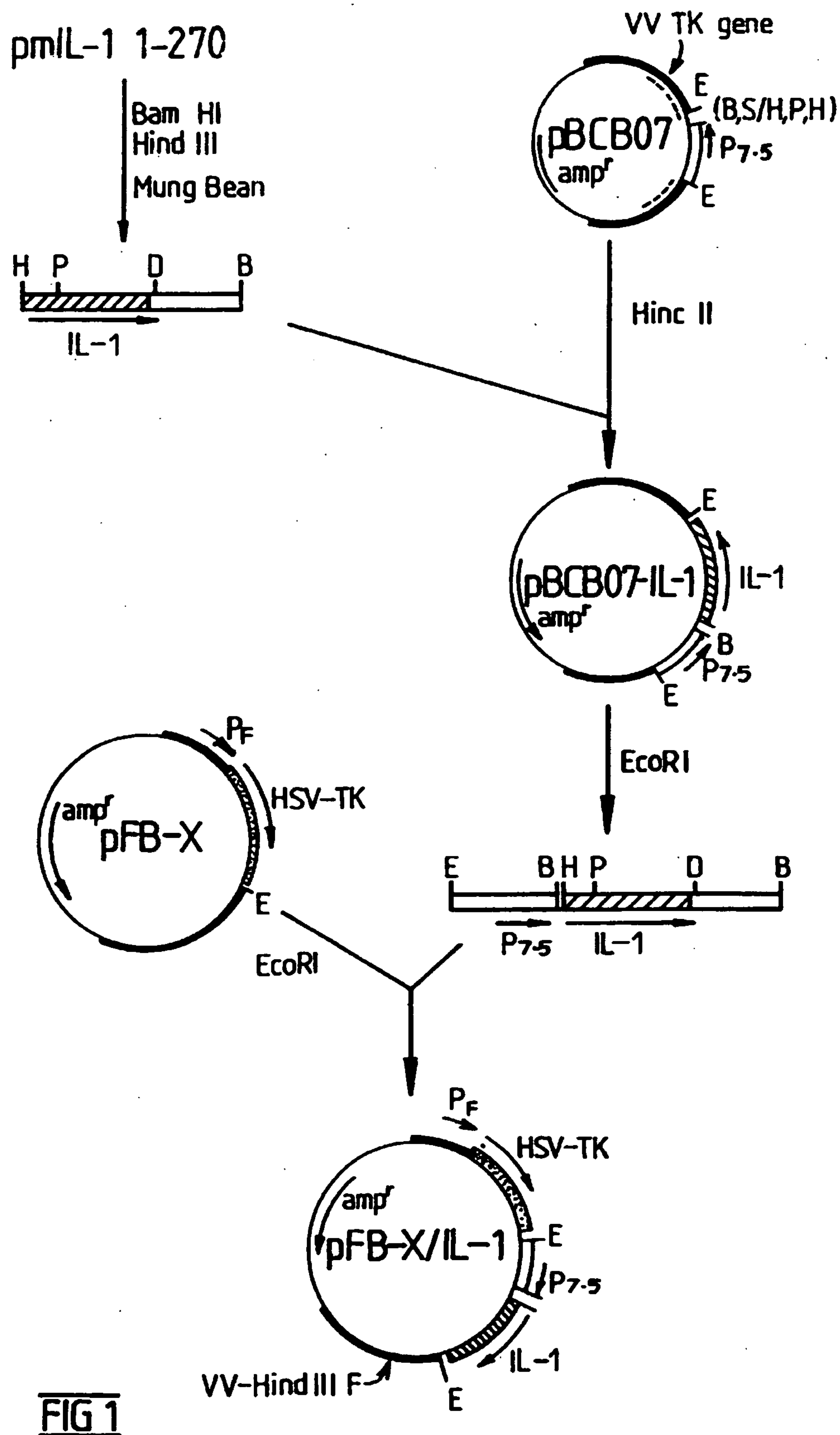

pmIL-1 1-270
Bam HI
Hind III
Mung Bean
H P D B
IL-1
VV TK gene
E
(B,S/H,P,H)
pBCB07
amp^r
P7·5
E
Hinc II
pBCB07-IL-1
amp^r
E
IL-1
B
P7·5
E
EcoRI
E B H P D B
P7·5 IL-1
PF
HSV-TK
amp^r
pFB-X
E
EcoRI
PF
HSV-TK
amp^r
pFB-X/IL-1
E
P7·5
IL-1
VV-Hind III F
E

FIG 1

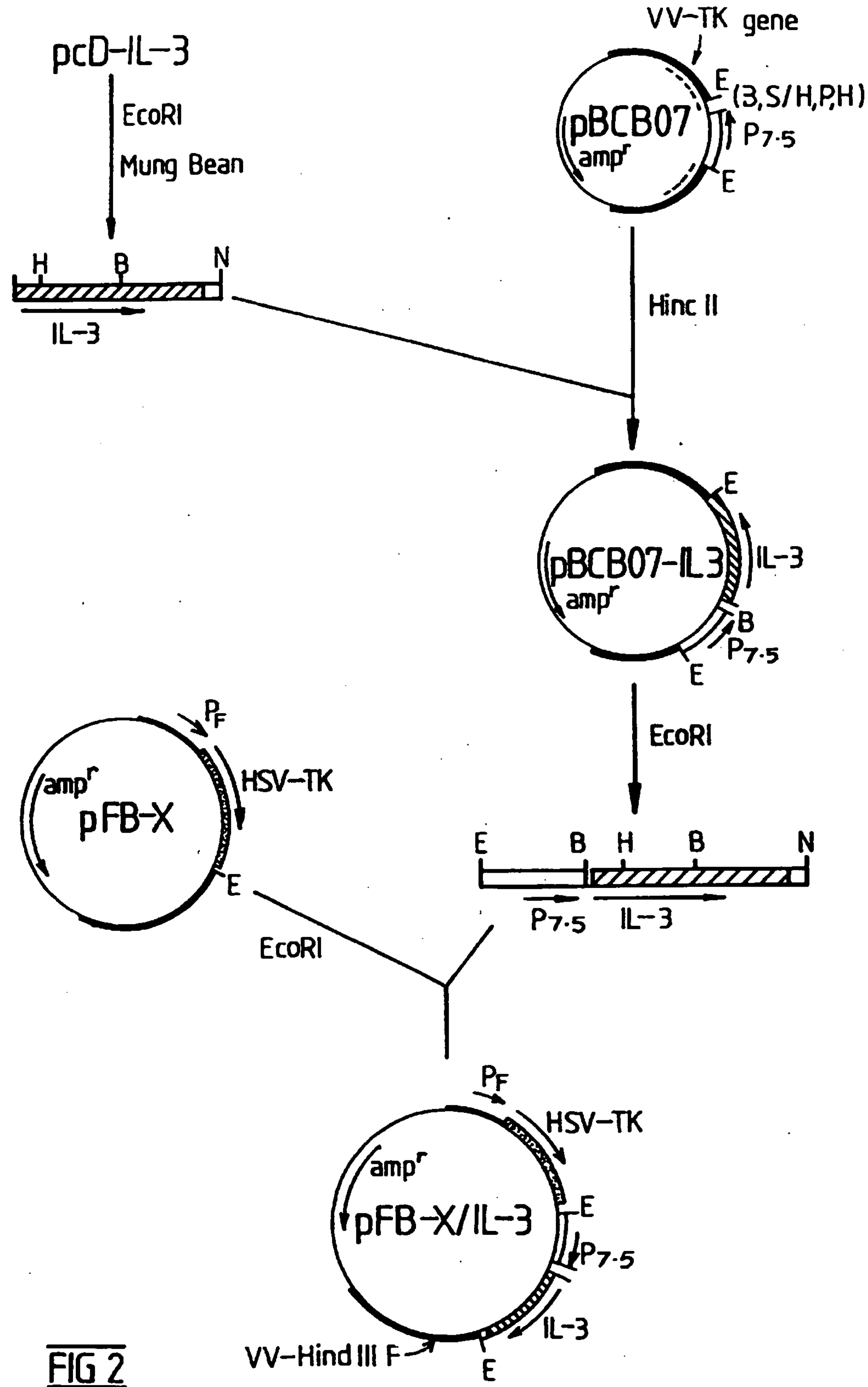

pcD-IL-3
EcoRI
Mung Bean
H
B
N
IL-3
VV-TK gene
E
(3,S/H,P,H)
pBCB07
amp^r
P7·5
E
Hinc II
E
pBCB07-IL3
IL-3
amp^r
B
P7·5
E
EcoRI
P_F
HSV-TK
amp^r
pFB-X
E
E
B
H
B
N
P7·5
IL-3
EcoRI
P_F
HSV-TK
amp^r
pFB-X/IL-3
E
P7·5
IL-3
VV-Hind III F
E

FIG 2

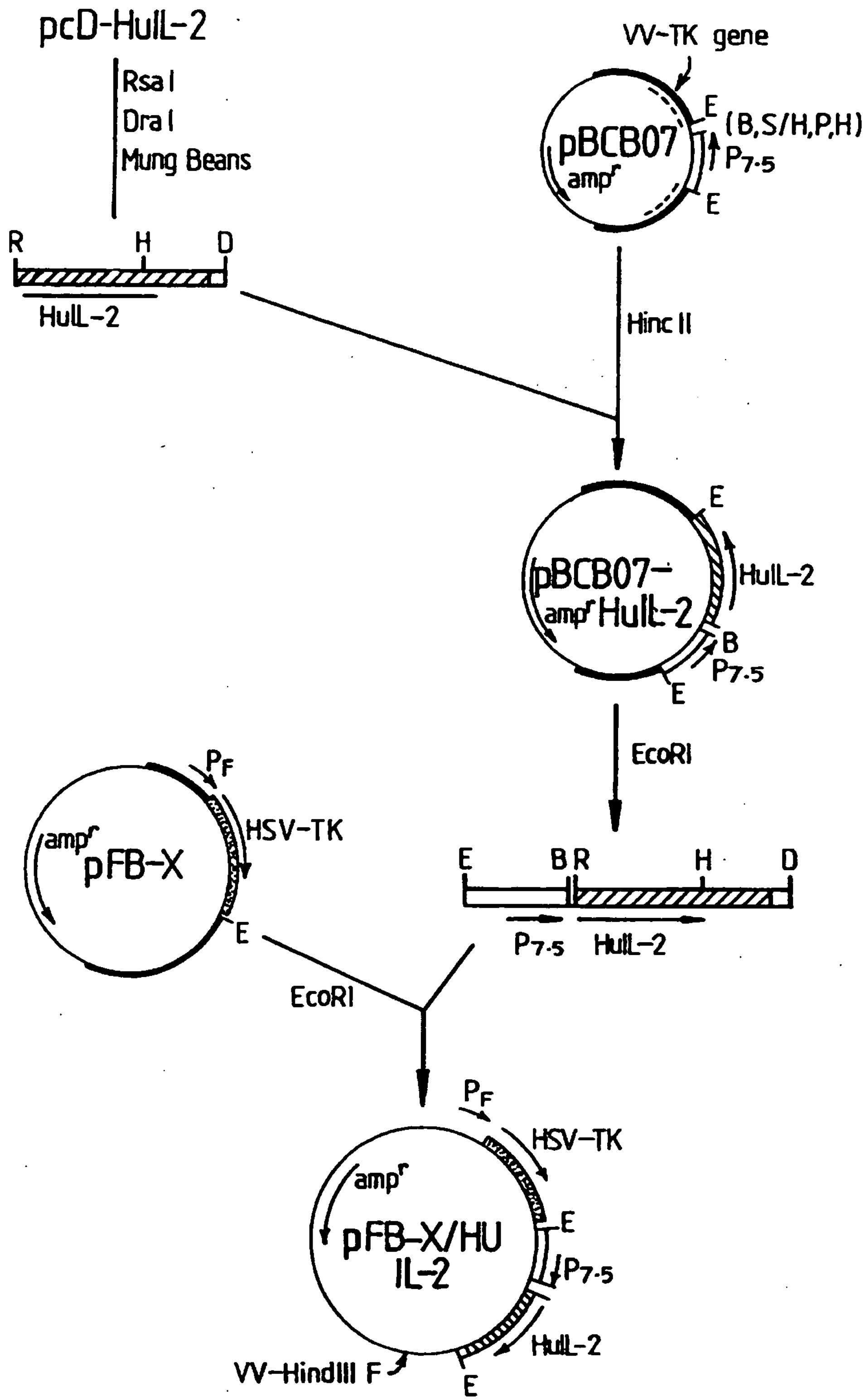

pcD-HuIL-2
Rsa I
Dra I
Mung Beans
R
H
D
HuIL-2
VV-TK gene
E
(B,S/H,P,H)
pBCB07
amp^r
P7·5
E
Hinc II
pBCB07-HuIL-2
amp^r
E
HuIL-2
B
P7·5
E
EcoRI
pFB-X
amp^r
PF
HSV-TK
E
E
B R
H
D
P7·5
HuIL-2
EcoRI
pFB-X/HU IL-2
amp^r
PF
HSV-TK
E
P7·5
HuIL-2
E
VV-HindIII F

FIG 3

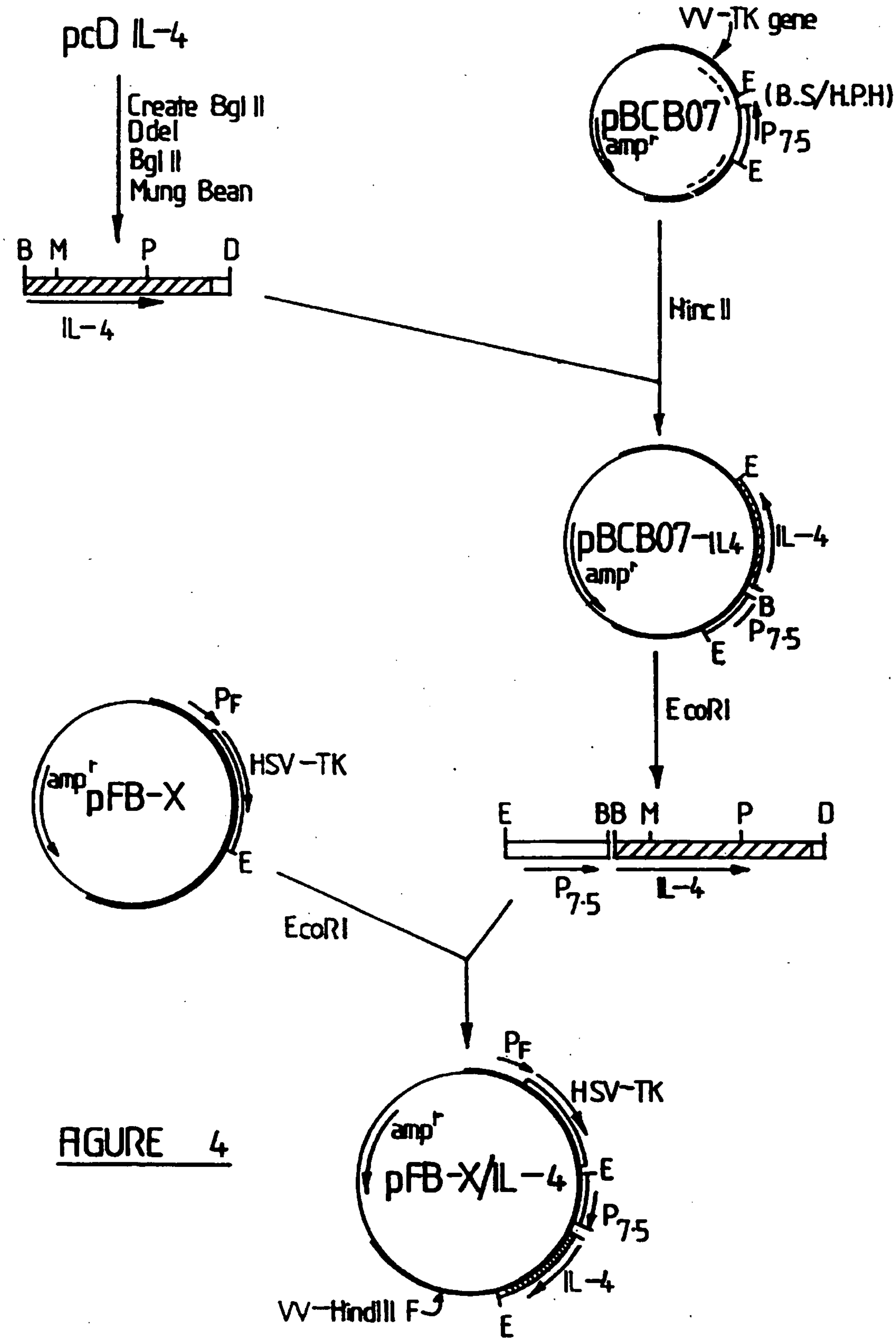

pcD IL-4
Create Bgl II
DdeI
Bgl II
Mung Bean
B M P D
IL-4
VV-TK gene
pBCB07
amp^r
E (B.S/H.P.H)
$P_{7.5}$
E
Hinc II
pBCB07-IL4
E
IL-4
B
$P_{7.5}$
E
EcoRI
P_F
HSV-TK
amp^r pFB-X
E
E BB M P D
$P_{7.5}$
IL-4
EcoRI
P_F
HSV-TK
amp^r
pFB-X/IL-4
E
$P_{7.5}$
IL-4
VV-HindIII F
E

FIGURE 4

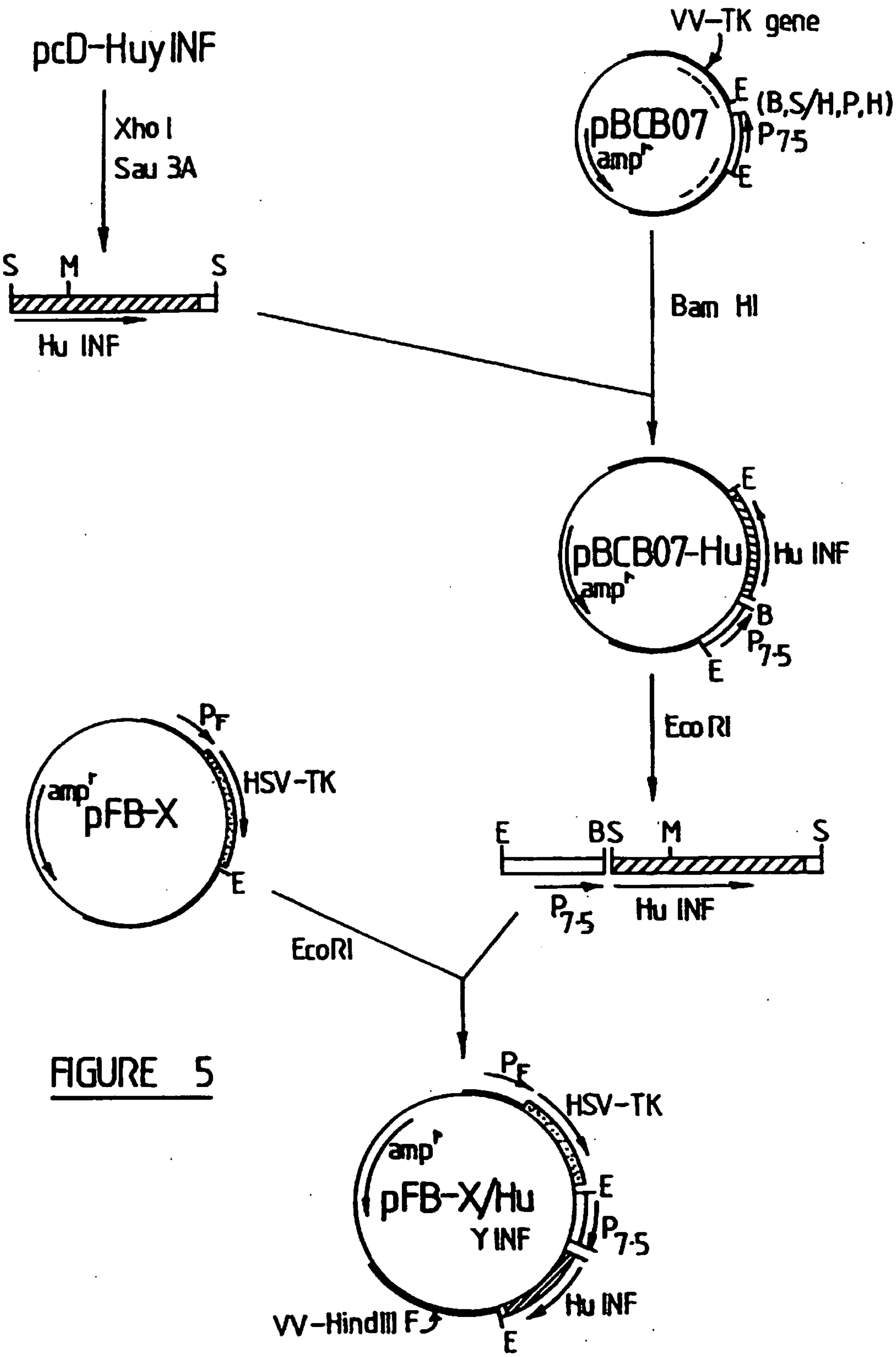

pcD-HuyINF
Xho I
Sau 3A
S
M
S
Hu INF
VV-TK gene
E
(B,S/H,P,H)
pBCB07
amp^r
P7.5
E
Bam HI
E
pBCB07-Hu
amp^r
Hu INF
B
P7.5
E
Eco RI
amp^r
pFB-X
P_F
HSV-TK
E
E
BS
M
S
P7.5
Hu INF
EcoRI
P_F
HSV-TK
amp^r
pFB-X/Hu
Y INF
E
P7.5
Hu INF
VV-HindIII F
E

FIGURE 5

FIG 6a

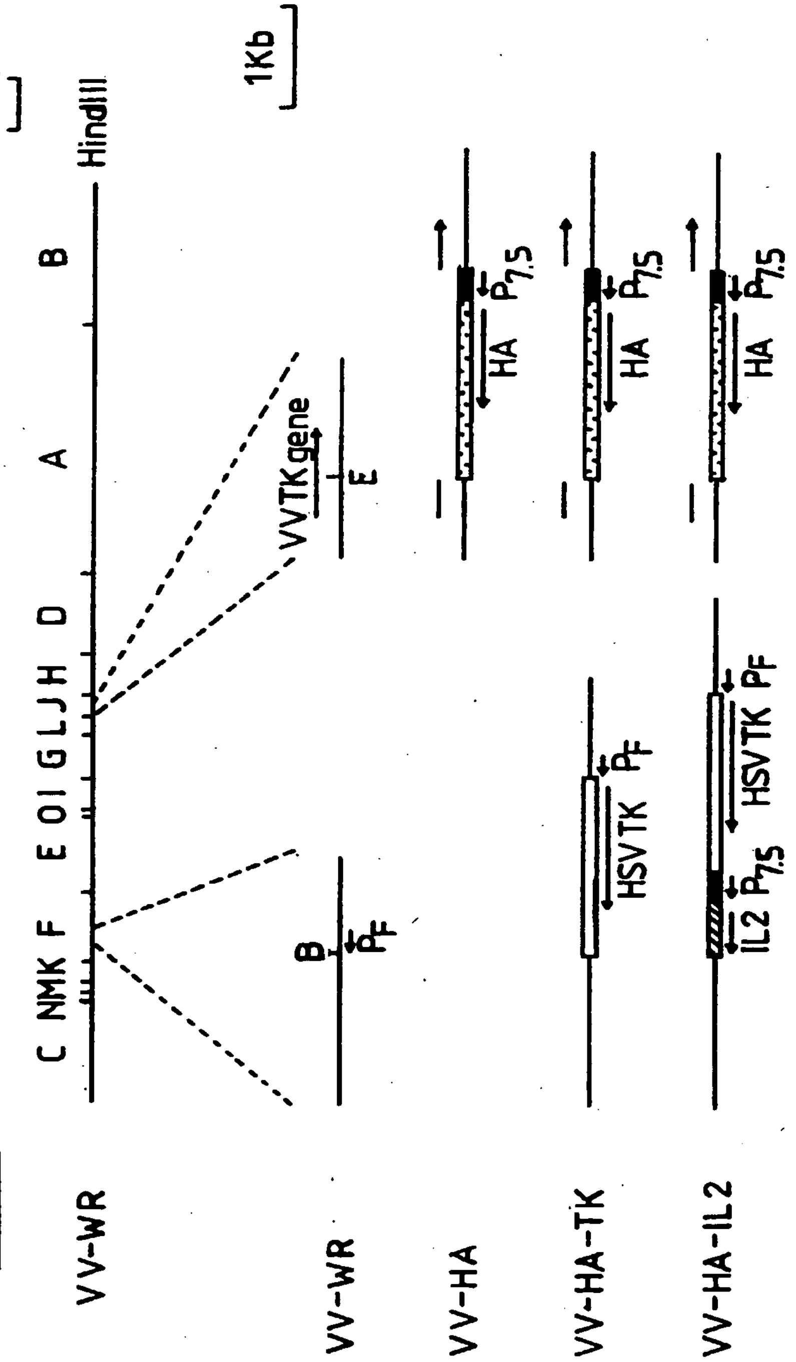
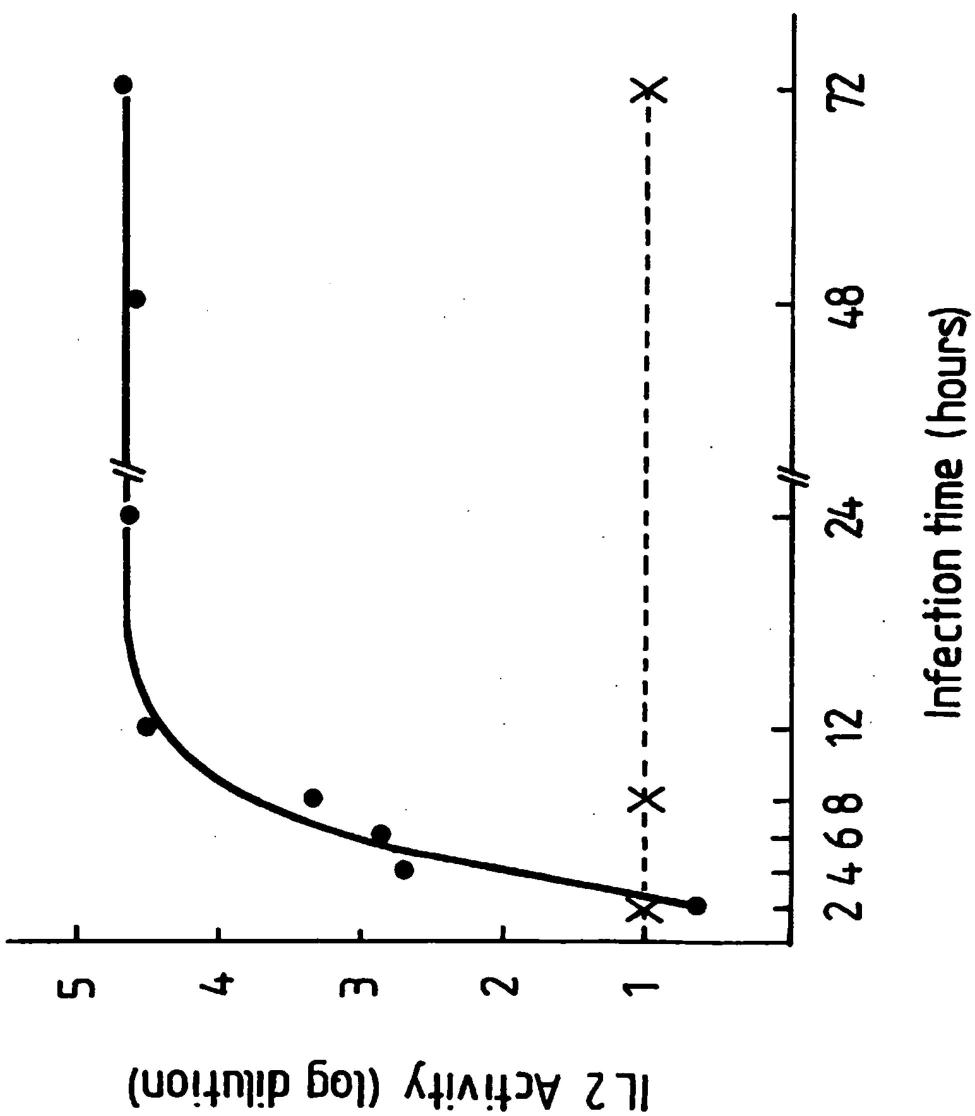
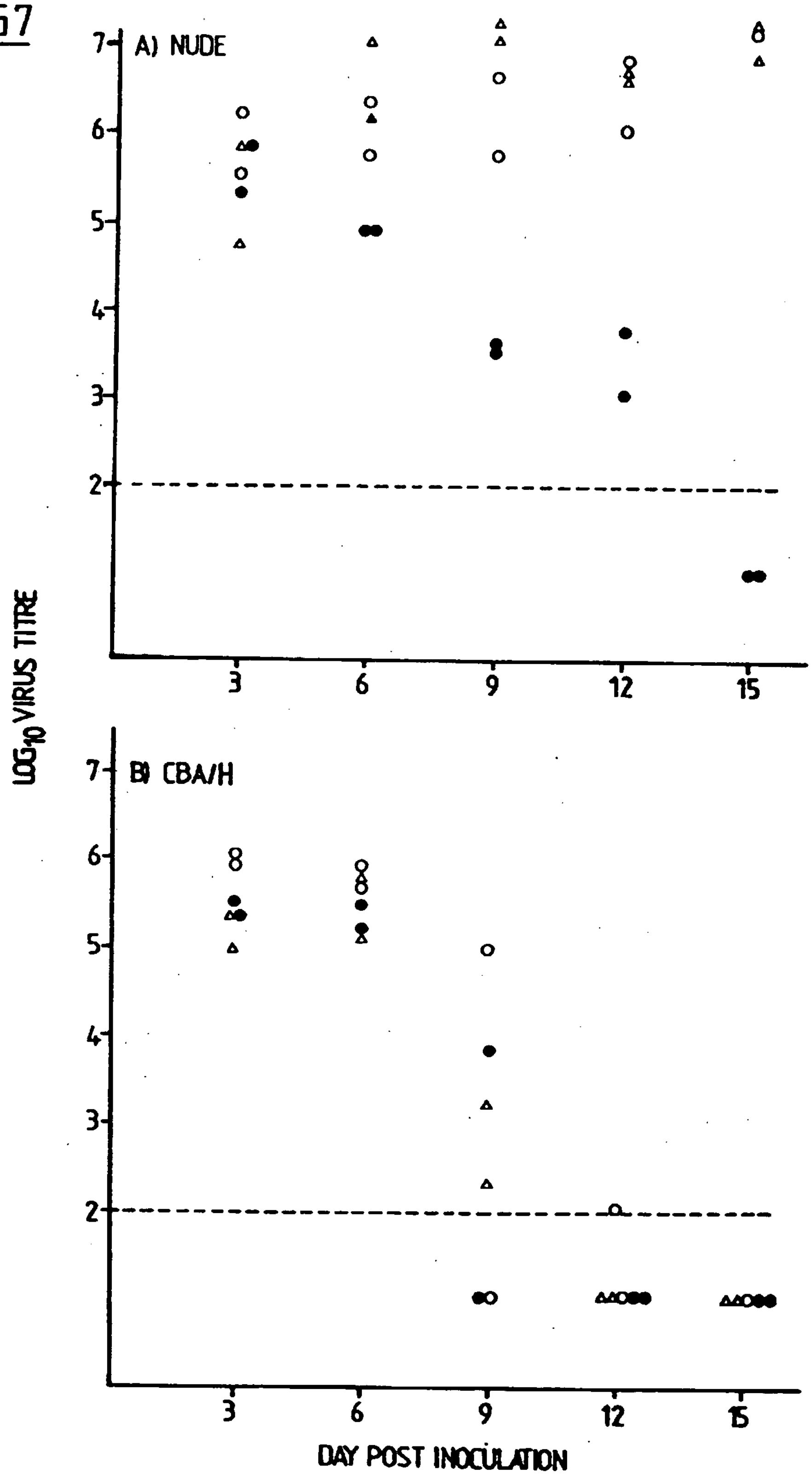

10 Kb
HindIII
1Kb
C NMK F E OI G L J H D A B
VV-WR
VV-WR
VV-HA
VV-HA-TK
VV-HA-IL2
VVTK gene
B
E
P_F
HA
P7.5
HSVTK
IL2

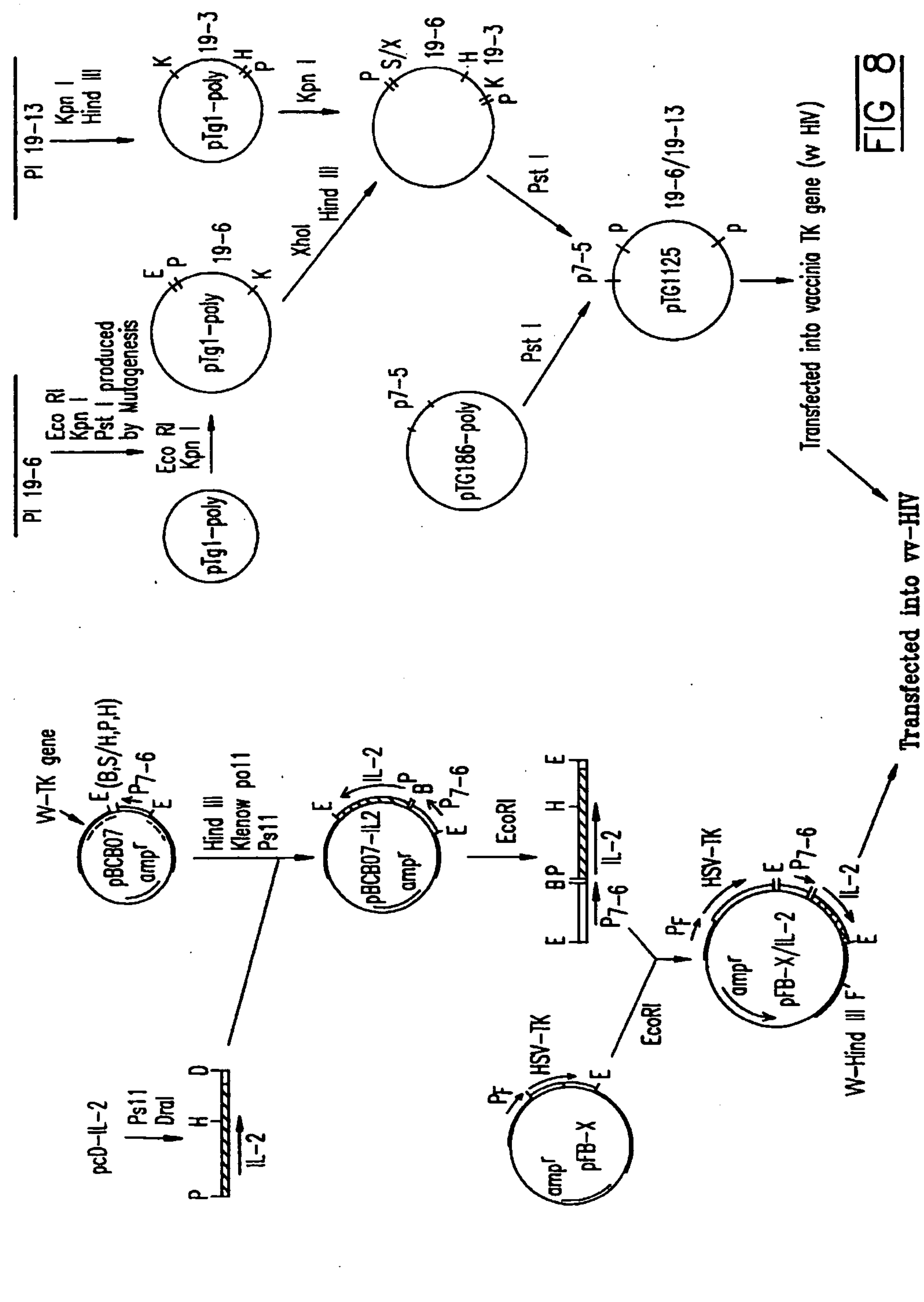
FIG 6b



IL 2 Activity (log dilution)
1
2
3
4
5
Infection time (hours)
2 4 6 8
12
24
48
72

FIG 7

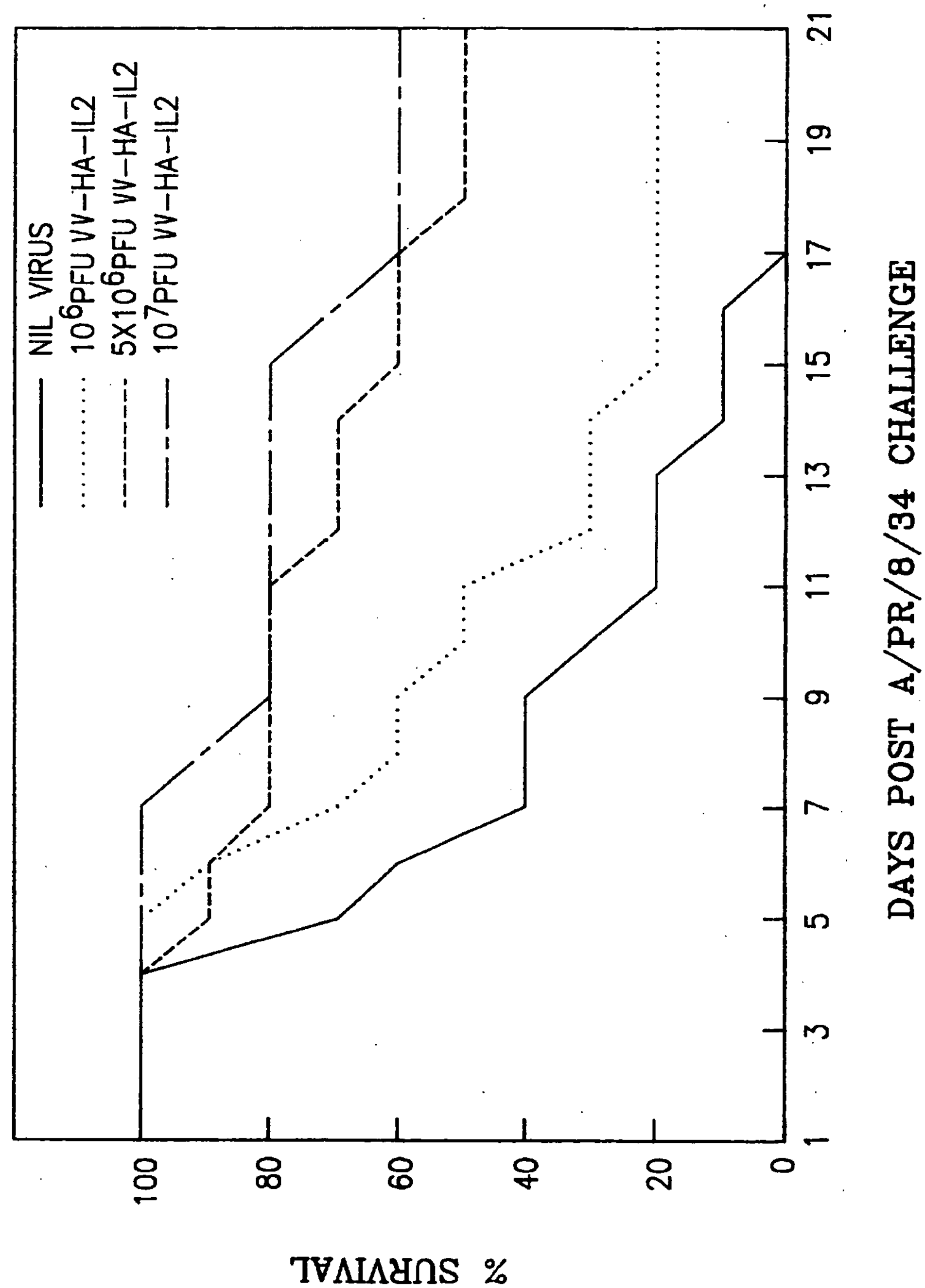

A) NUDE
B) CBA/H
LOG10 VIRUS TITRE
7
6
5
4
3
2
3
6
9
12
15
DAY POST INOCULATION



Pl 19-6
Eco RI
Kpn I
Pst I produced
by Mutagenesis
Eco RI
Kpn I
pTg1-poly
19-6
Pl 19-13
Kpn I
Hind III
19-3
Kpn I
XhoI
Hind III
S/X
Pst I
p7-5
pTG186-poly
pTG1125
19-6/19-13
Transfected into vaccinia TK gene (w HIV)
Transfected into vv-HIV
pcD-IL-2
Ps11
DraI
IL-2
W-TK gene
pBCB07
amp^r
Hind III
Klenow po11
Ps11
pBCB07-IL2
EcoRI
P7-6
HSV-TK
pFB-X
pFB-X/IL-2
W-Hind III F'

FIG 8

FIG. 9


NIL VIRUS
10^6PFU VV–HA–IL2
$5X10^6$PFU VV–HA–IL2
10^7PFU VV–HA–IL2
% SURVIVAL
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
DAYS POST A/PR/8/34 CHALLENGE

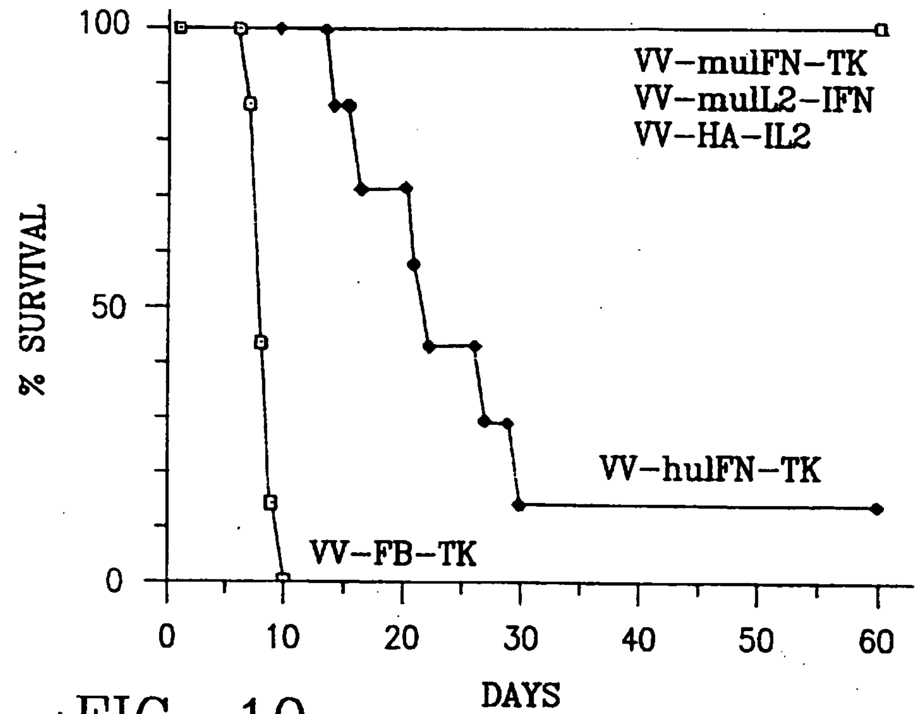

100
50
0
% SURVIVAL
VV-muIFN-TK
VV-muIL2-IFN
VV-HA-IL2
VV-huIFN-TK
VV-FB-TK
0
10
20
30
40
50
60
DAYS

FIG. 10

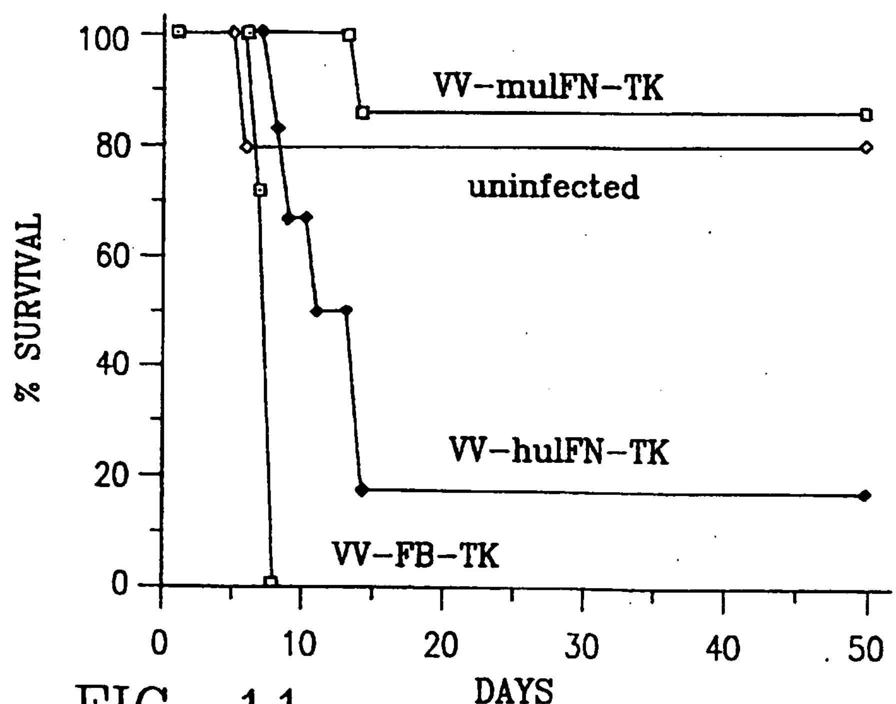

100
80
60
40
20
0
% SURVIVAL
VV-muIFN-TK
uninfected
VV-huIFN-TK
VV-FB-TK
0
10
20
30
40
50
DAYS

FIG. 11

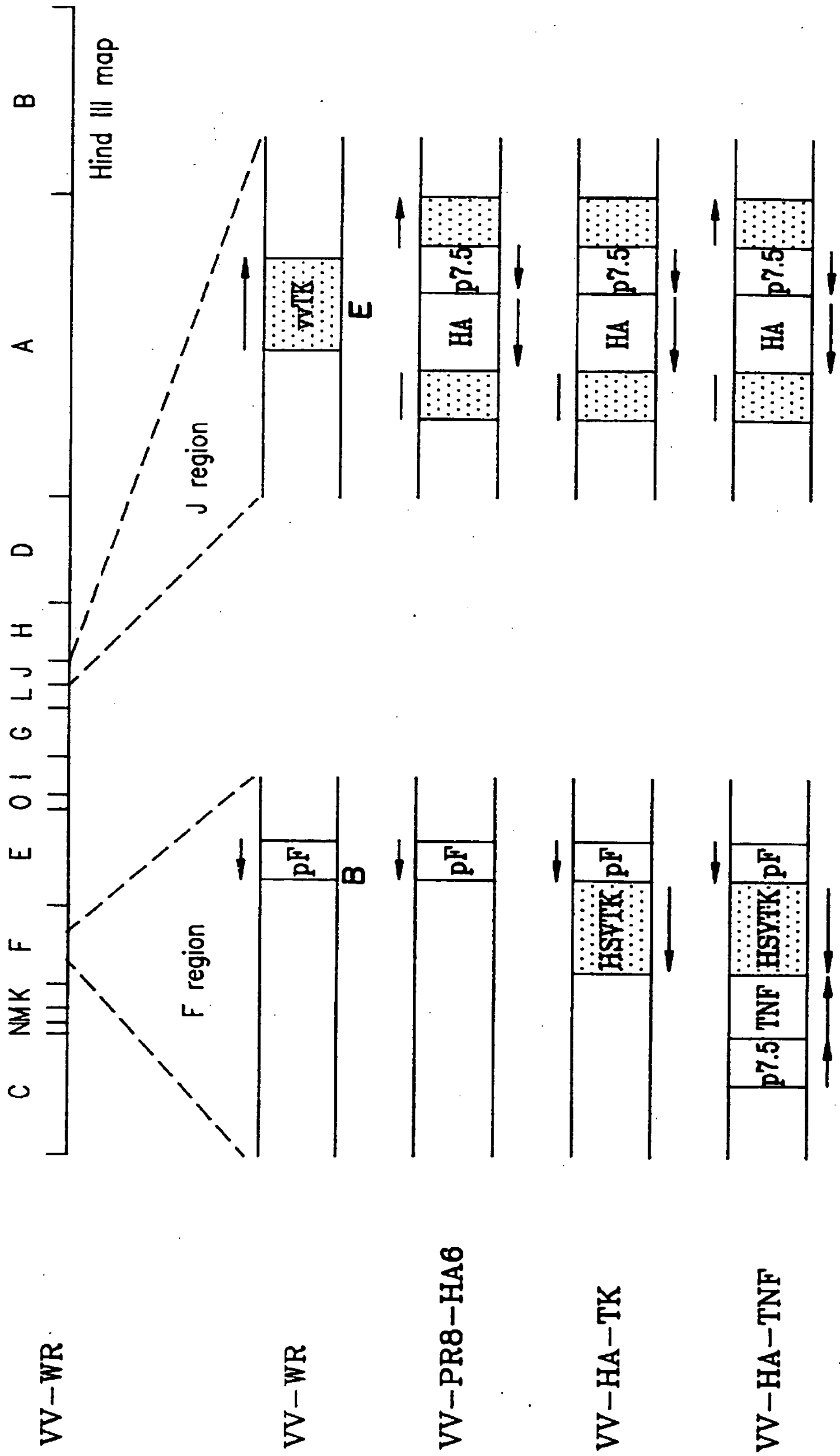

VV-WR
Hind III map
C
NMK
F
E
O
I
G
L
J
H
D
A
B
F region
J region
VV-WR
pF
B
vvTK
E
VV-PR8-HA6
pF
HA
p7.5
VV-HA-TK
HSVTK
pF
HA
p7.5
VV-HA-TNF
p7.5
TNF
HSVTK
pF
HA
p7.5

FIG. 12(a)

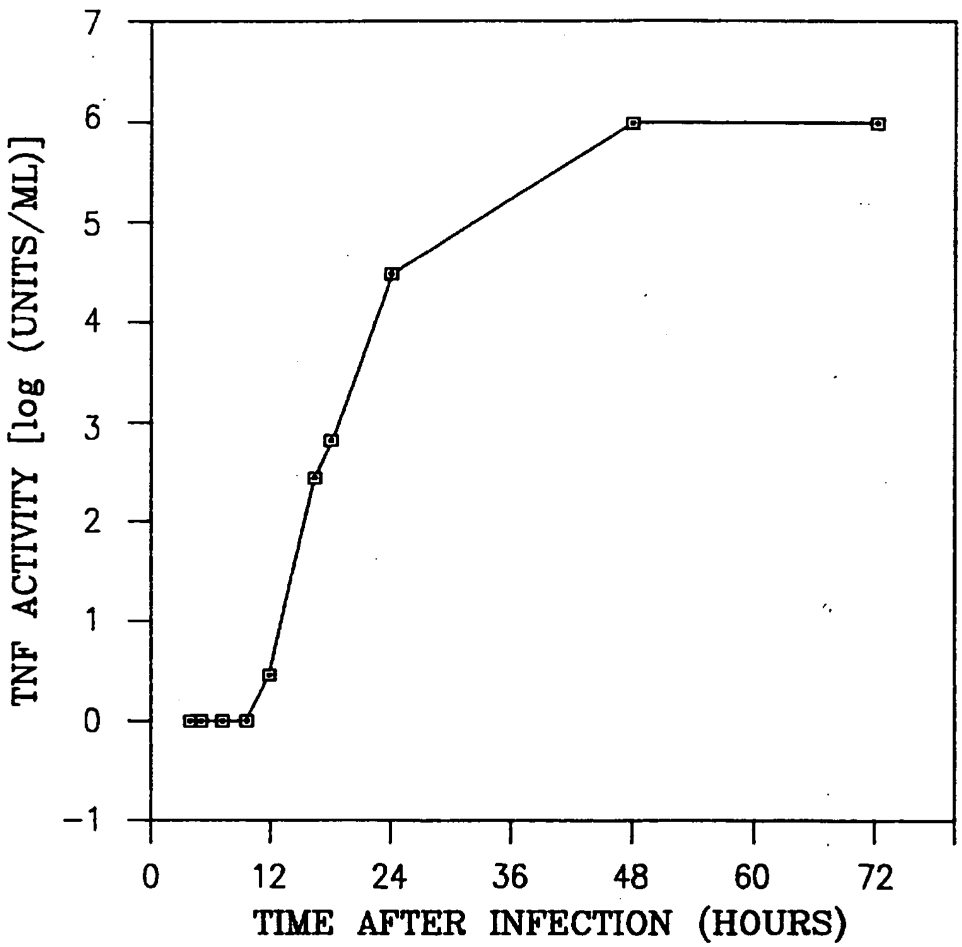

TNF ACTIVITY [log (UNITS/ML)]
7
6
5
4
3
2
1
0
−1
0
12
24
36
48
60
72
TIME AFTER INFECTION (HOURS)

FIG. 12(b)

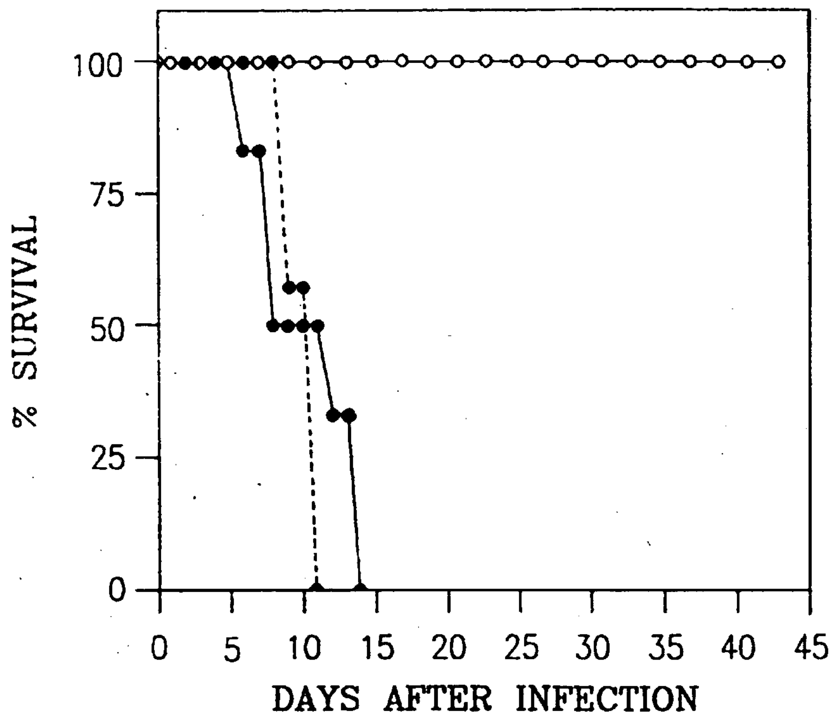

% SURVIVAL
100
75
50
25
0
0 5 10 15 20 25 30 35 40 45
DAYS AFTER INFECTION

FIG. 13(a)

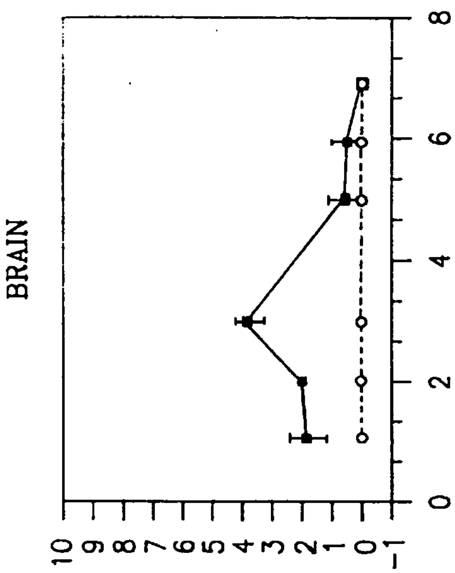


FIG. 13(c)

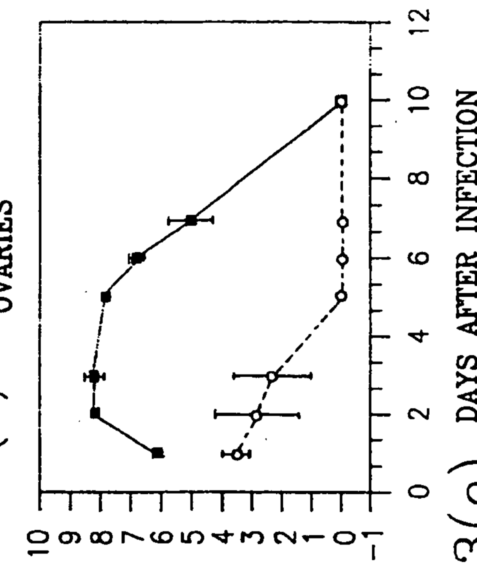


FIG. 13(e)

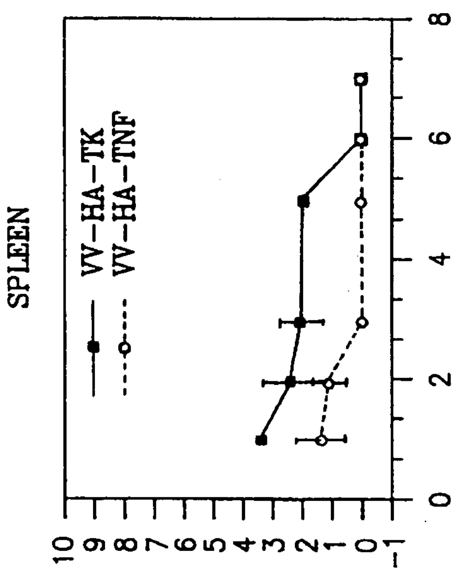


FIG. 13(b)

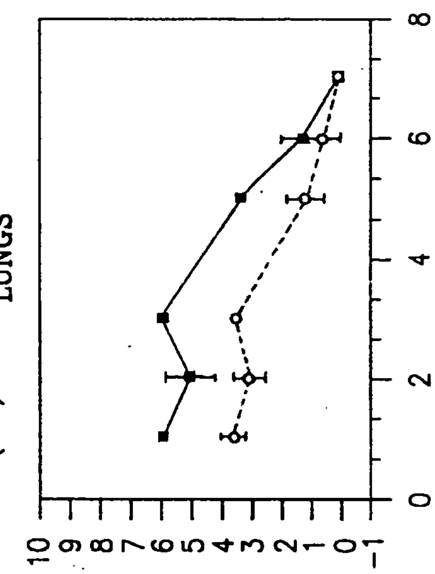


FIG. 13(d)

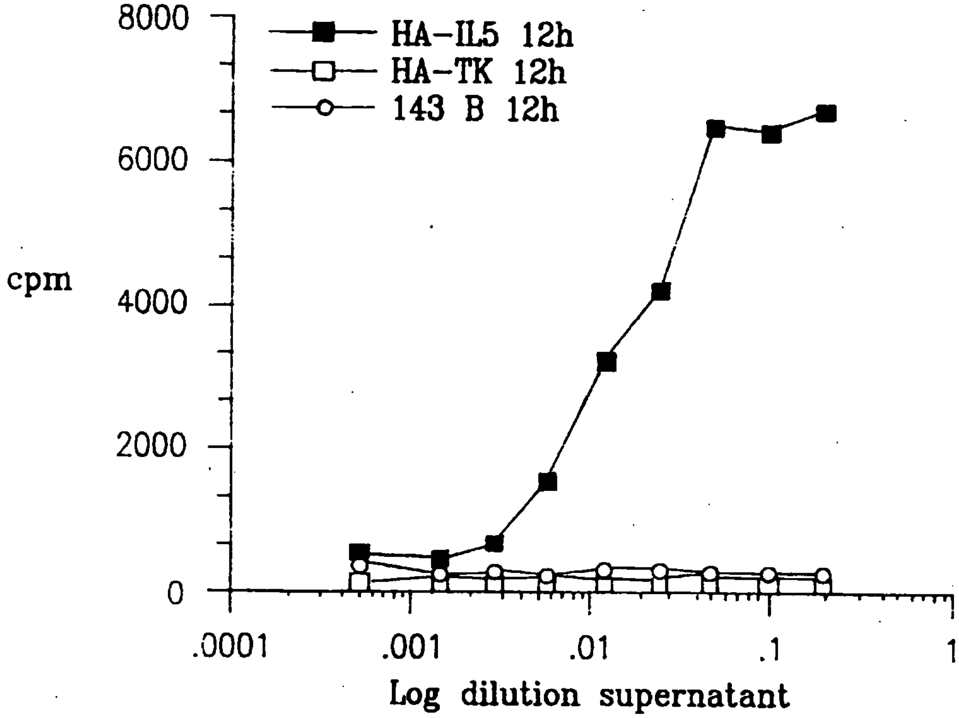

8000
6000
4000
2000
0
cpm
HA-IL5 12h
HA-TK 12h
143 B 12h
.0001
.001
.01
.1
1
Log dilution supernatant

FIG. 14

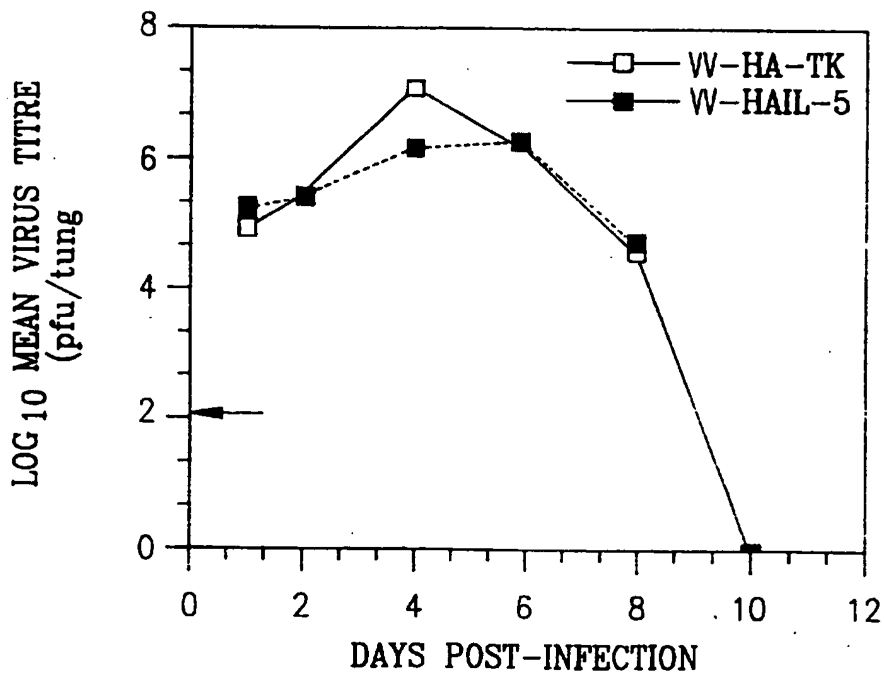
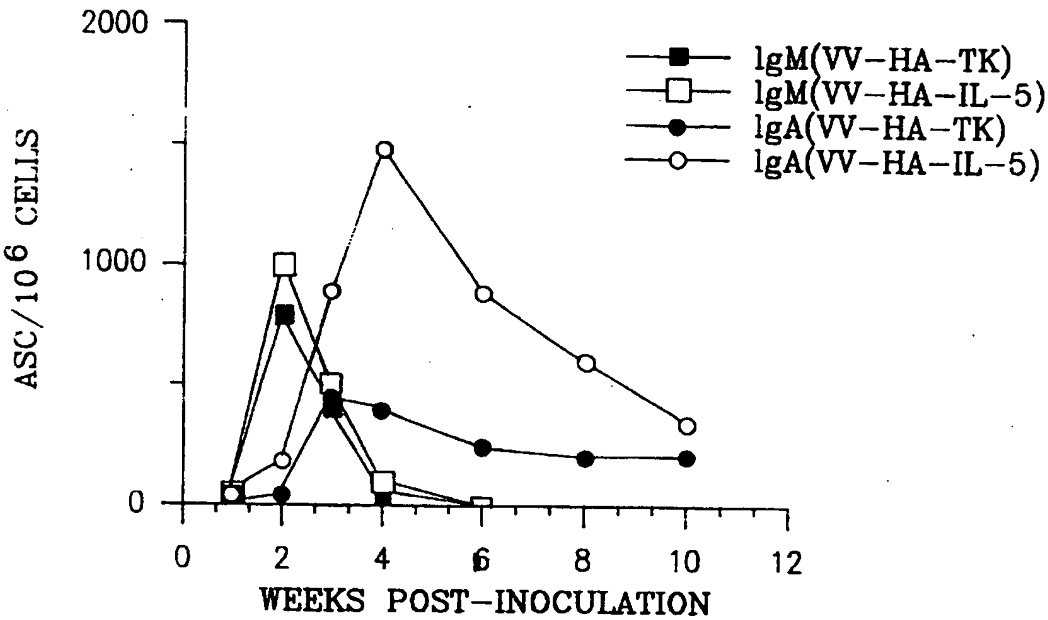

8
6
4
2
0
LOG 10 MEAN VIRUS TITRE (pfu/lung)
W-HA-TK
W-HAIL-5
0
2
4
6
8
10
12
DAYS POST-INFECTION

FIG. 15



2000
1000
0
ASC/10^6 CELLS
0
2
4
6
8
10
12
WEEKS POST-INOCULATION
lgM(VV-HA-TK)
lgM(VV-HA-IL-5)
lgA(VV-HA-TK)
lgA(VV-HA-IL-5)

FIG. 16

RECOMBINANT VACCINE

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 07/498,420 filed on Mar. 26, 1990, now abandoned; which is a continuation of application Ser. No. 07/203,060, filed on Jun. 1, 1988, now abandoned; which is a continuation application of international application Ser. No. PCT/AU87/00246, filed on Jul. 31, 1987.

BACKGROUND TO THE INVENTION

This invention concerns new vaccines developed using recombinant DNA technology to provide useful immune responses in circumstances where traditional vaccines may not be sufficiently effective.

Many existing live or killed vaccines are not without disadvantages, often significant, in respect of, for example, high production costs, poor response, low response to poorly immunogenic antigens, instability and a requirement for adjuvants. Furthermore, alternative vaccine preparations based on agents such as purified proteins or synthetic peptide antigens frequently offer only poor protection. In response to these problems, attention has turned to the development of vaccines in which recombinant DNA methods have been used to introduce antigens to which immunity is required, into carrier viruses such as vaccinia.

The advantage of the recombinant DNA approach is that an infectious recombinant virus simultaneously synthesises the foreign polypeptide and viral antigen, which can then be delivered to a host immune system as a superficial skin lesion. Vaccinia viruses have, for example, been modified for expression of the genes for hepatitis B, human immunodeficiency virus, influenza and malaria antigens; the construction of recombinant viruses carrying other antigens of medical or veterinary importance is under investigation.

In some instances, however, the immune response of recombinant vaccines may be of limited nature and magnitude. Thus, while peripheral immunisation with vaccinia-influenza recombinants provides good protection against lower respiratory tract infection, it fails to induce immunity in the upper respiratory tract. On the other hand, peripheral immunisation with recombinant vaccines may prove ineffective when local rather than systemic immunity is required, as in say the gastro-intestinal tract.

There have been various attempts to remedy these deficiencies, including expression of vaccine antigens through viruses having stronger promoters, such as poxvirus, but to date these have not met with significant success. The present invention provides an effective means for enhancing the immune response to the specific foreign antigenic polypeptides of recombinant vaccines.

The immune system is regulated in part by molecules, known as lymphokines, which are released by lymphocytes and help or modify the functions of other classes of lymphocytes. The present invention is based on a recognition that the expression of appropriate lymphokines from recombinant bacterial or viral vaccines can boost and/or modify the immune response to viral, bacterial or co-expressed foreign antigenic polypeptides.

SUMMARY OF THE INVENTION

Accordingly, in its broadest aspect, this invention provides a recombinant vaccine comprising a vaccine vector which incorporates a first nucleotide sequence capable of being expressed as an antigenic polypeptide, together with a second nucleotide sequence capable of being expressed as all or an active part of a lymphokine effective in enhancing or modifying the immune response to the antigenic polypeptide.

In accordance with one embodiment of this invention, the first nucleotide sequence capable of being expressed as an antigenic polypeptide may be a "native" sequence of the host vector itself, for example vaccinia or herpes virus. In this embodiment, administration of the vaccine of this invention provides augmentation and/or selective induction of the immune response to the "native" antigenic polypeptide. In other words, the inclusion of the lymphokine in the recombinant vaccine may substantially modify the immunogenicity of the host vector. Where the host vector is a virus such as vaccinia virus, the system offers the advantage that the lymphokine is continuously produced at the site of virus replication throughout the immune response, with the effect in some instances of dramatic modification of the pathogenicity of the vaccinia virus, and in other cases altering the immune response to the viral antigens.

In another embodiment, the vaccine vector incorporates a nucleotide sequence capable of being expressed as an antigenic polypeptide which is foreign or heterologous to the host vector. In this embodiment, administration of the vaccine to an individual will result in augmentation and/or selective induction of the immune response of the individual to both antigenic polypeptide of the host vector and to co-expressed foreign or heterologous antigenic polypeptide. As discussed below, the co-expression of the lymphokine with the antigenic polypeptide(s) ensures that on administration of the vaccine the lymphokine and antigenic polypeptide(s) are delivered together at the same time and at the same site, giving an improved immune response to the antigenic polypeptide(s).

In another aspect, the present invention provides a method for producing an immune response in a human or animal, in particular an immunodeficient or immunosuppressed human or animal, which comprises the step of administering to the human or animal a recombinant vaccine as broadly described above.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow chart which depicts the construction of vaccinia virus expressing IL-1.

FIG. 2 is a flow chart which depicts construction of a vaccinia virus expressing IL-3.

FIG. 3 is a flow chart which depicts construction of a vaccinia virus expressing HuIL-2.

FIG. 4 is a flow chart which depicts construction of a vaccinia virus expressing IL-4.

FIG. 5 is a flow chart which depicts construction of a vaccinia virus expressing; Hu INF.

FIG. 6*a* depicts the genomic configuration of VV recombinants.

FIG. 6*b* is a graph which shows the production of IL2 production by VV-HA IL2-infected human 143B cells over a period of time.

FIG. 7 shows the growth of vaccinia virus recombinants in the foot pads of athymic Swiss outbred nude mice and athymic CBA/H mice.

FIG. 8 is a flow chart which depicts the construction of human immunodeficiency virus IL-2 recombinant vaccinia virus in accordance with the present invention.

FIG. 9 is a graph which shows the percent survival over a period of time for groups of Swiss outbred nude mice

which were inoculated with VV-HA-IL2 at various doses and which were challenged after 30 days with 10 LD_{50} of A/PR,8/34; and a control group which was not given any virus.

FIG. 10 is a graph which shows the survival over a period of time of athymic Swiss outbred nude mice infected with VV recombinants.

FIG. 11 is a graph which shows the percent survival over a period of time of sublethally irradiated CBA/H euthymic mice infected with VV recombinants.

FIG. 12*a* shows the genomic configuration of VV recombinants.

FIG. 12*b* is a graph which shows the expression of biologically active INF over a period of time by VV-HA-TNF-infected cells.

FIG. 13*a* is a graph which shows the percent survival over a period of time with respect to immunodeficient mice which have been inoculated with VV-recombinants.

FIGS. 13*b–e* are a group of graphs which show the growth kinetics of VV recombinants as determined by samples taken from selected organs of mice which were injected with VV-HA-TNF or VV-HA-TK.

FIG. 14 is a graph which shows that 12 hour supernatants from VV-HA-IL5-infected 143B cells stimulated BCL_1 to proliferate in a dose-dependent manner.

FIG. 15 is a graph which shows the mean virus titre over a period of time after infection with VV-HA-IL5 and VV-HA-TK.

FIG. 16 is a graph which shows the number of specific antibody-secreting cells (ASC) as a function of time after intranasal inoculation with 10^7 plaque-fonning units of virus at day 0.

It will be appreciated from the broad description set out above that the present invention has particular application in the augmentation of immune responses in immunodeficient or immunosuppressed individuals. In one particularly important aspect of this invention, there is provided a recombinant vaccine for use in the treatment or prophylaxis of immunodeficient or immunosuppressed individuals infected with the human immunodeficiency virus (HIV), which comprises a vaccine vector which incorporates a nucleotide sequence capable of being expressed as an antigenic polypeptide derived from the human immunodeficiency virus (HIV), together with a second nucleotide sequence capable of being expressed as all or an active part a lymphokine effective in enhancing or modifying the immune response of the individual to the HIV antigenic polypeptide.

The lymphokines which may be expressed in vaccines according to this invention include those designated interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), tumour necrosis factor (TNF), and γ-interferon (γ-IFN).

Interleukin-1 is a peptide hormone largely produced by activated macrophages. IL-1 modulates the proliferation, maturation and functional activation of a broad spectrum of cell types (1–3) and plays a major role in the initiation and amplification of immune and inflammatory responses through its action on these diverse cell populations (4). The gene for murine (5) and human (6) IL-1 has been cloned and expressed in *E.coli*.

Interleukin-2 is a lymphokine produced by helper T cells and is active in controlling the magnitude and type of the immune response (7). Other functions have also been ascribed to IL-2 including the activation of NK cells (8) and the stimulation of cell division in large granular lymphocytes and B cells (9). Numerous studies in mice and humans have demonstrated that deficient immune responsiveness both in vivo and in vitro can be augmented by IL-2. For example, exogenous IL-2 can restore the immune response in cyclophosphamide-induced immunosuppressed mice (10) and athymic (nude) mice (11). Furthermore, IL-2 can restore responsiveness of lymphocytes from patients with various immunodeficiency states such as leprosy and cancer (12). IL-2 has also been used for the treatment of cancer (13). The gene for murine (14) and human (15) IL-2 has been cloned and sequenced.

Interleukin-3 is a hormone-like glycoprotein produced by lectin or antigen activated T lymphocytes and possibly other cells within the bone marrow. The hormone stimulates the growth and differentiation of haematopoietic progenitor cells and multipotential stem cells and has been described under a variety of names, among them multi-colony stimulating factor, and haematopoietic growth factor (16). The gene for mouse IL-3 has been cloned and sequenced (17).

Interleukin-4 is a T cell derived factor that acts as an induction factor on resting B cells, as a B cell differentiation factor and as a B cell growth factor (18). The factor also stimulates T cells and acts as a mast cell growth factor (18). The gene for murine (19) and human (20) IL-4 has been isolated and sequenced.

γ-interferon is also a T cell derived molecule which has profound effects on the immune response. The molecule promotes the production of immunoglobulin by activated B cells stimulated with interleukin-2. γ-interferon also increases the expression of histocompatibility antigens on cells which associate with viral antigens to stimulate cytotoxic T cells. The gene for human γ-interferon has been isolated and sequenced (21).

Other lymphokines including interleukin-5 and tumour necrosis factor are also well known. Thus, interleukin-5 (IL-5) stimulates the production of several immunoglobulin classes, however its major function may be to promote IgA synthesis, thereby playing a crucial role in regulating mucosal immune responses. The mechanism by which IL-5 acts is unclear, although in vitro data indicate that it promotes terminal B cell differentiation.

The co-expression of a lymphokine such as IL-2 and an antigenic polypeptide by a recombinant vaccine (such as a recombinant virus vaccine) ensures that they are produced together by the same infected cells in a very localised area. This can be expected to lead to an elevation and acceleration of response to the virus vector component of the vaccine, e.g. vaccinia virus, with attendant benefits such as a reduction in the risks of complication associated with the use of vaccinia virus in normal individuals, and to those unidentifiable individuals who react adversely to vaccinia virus. Also, where there are immunological defects, as in the case of patients suffering from AIDS, leprosy or cytomegalovirus infection, co-expression of lymphokine could be instrumental in overcoming the defects to allow a normal response to the antigenic polypeptide and/or vector virus.

Furthermore, it is anticipated that the present invention will prevent or at least minimise the complications such as generalised vaccinia that can occur when vaccine is administered inadvertently to immunodeficient recipients (32).

Further, cancer patients often show a negligible or poor immunological response to the cancer antigens. It may be possible to enhance those responses to useful levels by taking cancer cells from the hosts, infecting them with, say, vaccinia virus/IL-2 recombinants, and returning them to the

patient. To guard against generalised vaccinia infection or spread of the cancer cells it may, of course, be advisable to inactivate the recombinant-infected cancer cells prior to return to the patient.

Other lymphokines (e.g. IL-1, IL-3, IL-4, IL-5, TNF and γ-IFN), are involved in the control and augmentation of responses in other parts of the immune system including granulocyte-macrophage lineage, eosinophil differentiation and mucosal immunity. Construction of co-expressive vaccines will enable advantage to be taken of these specific modes of activity. Thus, as they are believed to have a role in the generation of protective responses at mucosal surfaces, such as in the gut, which promote expulsion of and immunity to parasites, a vaccine co-expressing IL-3 (or other lymphokinel with a helminth or other parasite antigenic polypeptide would be expected to give rise to enhanced immunity compared to that from the parasite antigen alone.

Whilst a specific example of co-expression of the influenza haemagglutinin (HA) is described in detail herein, it will be appreciated that the present invention may be applied for the co-expression of other foreign or heterologous antigens including hepatitis virus, herpes simplex virus, Epstein-Barr virus and human immunodeficiency virus (HIV) antigens, as well as malaria antigens. Prior work has demonstrated that protection may be obtained against influenza virus following immunization with a recombinant vaccinia virus that expresses influenza haemagglutinin (34,35). However this prior work does not teach or suggest the co-expression of the haemagglutinin and a lymphokine in the same recombinant vaccinia virus, or the advantages arising from such co-expression as disclosed herein.

As previously described, vaccinia virus has been used as a vaccine vector to deliver antigens of unrelated infectious agents such as hepatitis B virus (22) and human immunodeficiency virus (23). The expression of an inserted gene in vaccinia virus requires that the gene be placed next to a vaccinia promoter. The promoter usually used is designated p7.5 (22). This chimeric gene is then placed next to a DNA fragment of vaccinia virus taken from a non-essential region of the virus. Insertion into infectious virus is by homologous recombination in which a marker rescue is used to select for virus recombinants. By way of example, the marker rescue can be either selection for thymidine kinase negative (TK^-) virus in which the foreign gene has been inserted and thereby inactivating the TK gene; or by selecting for TK^+ virus in which the foreign gene is flanked by the herpes simplex TK gene. The latter is generally used to construct double recombinants that is, viruses expressing two foreign genes.

The expression of lymphokine genes in vaccinia virus may be detailed as two stages; the first is to create a plasmid in which the lymphokine is under the control of a vaccinia promoter 7.5 and downstream from a thymidine kinase (HSV) gene. This plasmid is then used *to transfect TK−* cells previously infected with a TK^- vaccinia virus expressing another foreign gene. TK^+ recombinant virus is then selected by culturing cells in the presence of methotrexate.

Although this invention has primarily been described with reference to vaccinia virus as the vaccine vector, it is to be understood that the inventive concept resides in co-expression of an antigenic polypeptide and lymphokine, and this concept may be realised using other vaccine vectors, such as other poxvirus, herpes virus, adenovirus or bacteria.

It is also to be understood that the invention is not limited by application to man or other species specifically mentioned herein, but may find application in a wide range of animal species.

Methods for construction and testing of recombinant vaccines according to this invention will be well known to those skilled in the art, however, for better understanding of the invention some typical techniques will now be described. Standard procedures for endonuclease digestion, ligation and electrophoresis were carried out in accordance with the manufacturer's or supplier's instructions. Standard techniques are not described in detail and will be well understood by persons skilled in the art.

EXAMPLE 1

Plasmids containing IL-1, IL-2, IL-3, IL-4 and γ-interferon are shown in FIGS. 1–5. Plasmids pmIL-1 1.270, pcD-IL-3, pcD-HuIL-2, pcD-IL-4 and pcD-HuγINF were obtained from DNAX Research Institute. The excised coding sequence is shown as the hatched bar. It is necessary to use different restriction endonucleases to create suitable termini for insertion into plasmid; these are detailed in the respective diagrams. pBCB07 (25,38) contains the vaccinia 7.5K promoter, PFB-X (27) contains the HSV TK coding sequence (stippled) inserted at a BamHl site downstream from a promoter in the vaccinia HindIII region. The recombinant plasmids pFB-X/IL1, pFB-X/IL2, pFB-X/IL3, pFB-X/IL4, pRB-X/γ-interferon contain HSV-TK and lymphokine genes 3' to different vaccinia virus promoters and with flanking sequences derived from the HindIII F fragment of vaccinia virus. The orientations of genes and promoters are shown with arrows, vaccinia virus sequences with solid lines and plasmid DNA by thin lines. The pFB- plasmids with inserted lymphokine genes are used to transfect 143B (TK^-) cells previously infected with a TK^- vaccinia virus according to conditions previously described (24). The site of insertion of HSV TK and lymphokine coding sequences and transposed vaccinia promoters in the vaccinia virus genome are shown in FIG. 6. Vaccinia-virus WR strain HindIII restriction fragments are shown in the top line. Lower lines show in expanded form the DNA configurations at insertion sites in the HindIII J and F fragments. Orientations of coding sequences and promoter sequences are shown with arrows.

EXAMPLE 2

This example describes the construction of a recombinant vaccinia virus (VV) expressing murine IL2 and the effect of the lymphokine on virus growth and immunogenicity.

FIG. 6 shows:

(a) Genomic configuration of VV recombinants. A HindIII map of VV WR strain is shown with insertion points at EcoRI (E) and BamHI (B) sites in the J and F fragments respectively. Arrows indicate orientations of VV TK gene, VV promoters and inserted influenza HA, HSV TK and murine IL2 coding sequences.

(b) Time course of IL2 production by VV-HA IL2-infected human 143B cells. IL2 activity in VV-HA-IL2-infected supernatants, circles and solid line; VV-HA or uninfected cell supernatants, crosses and dotted line.

FIG. 7 shows the growth of vaccinia virus recombinants in the foot pads of athymic Swiss outbred nude mice (a) and athymic CBA/H mice (b). 2×10^7 PFU of VV-HA (triangles), VV-HA-TK (open circles) or VV-HA-IL2 (closed circles) were injected subcutaneously in 20 μl into hind foot pads which were assayed for infectious virus on 143B cells on the indicated days. Points represent the titres of infectious virus present in individual mice.

As shown schematically in FIG. 6*a*, cDNA encoding murine IL2 (14) was inserted into the HindIII F region of a

VV recombinant, VV-HA (26), which already expressed the influenza haemagglutinin (HA). The IL2 recombinant virus, VV-HA-IL2, coexpressed HA and IL2 using the same VV 7.5 Kd promoter but from separate sites in the viral genome. Since the herpes simplex virus (HSV) thymidine kinase (TK) gene was used as a selectable marker for virus construction a control virus VV-HA-TK, expressing HSV TK but not IL2 was constructed. Significant levels of biologically active IL2 were detected in supernatants from human 143B cells infected with VV-HA-IL2 within 4 hours and reached maximum activity around 12 hours (FIG. *6b*).

Athymic nude mice were inoculated into the right hind footpad with VV-HA-IL2 or control virus (VV-HA-TK). VV-HA-IL2 induced a mild swelling in the foot which resolved after several days; in contrast VV-HA-TK produced a severe necrotic lesion that remained unresolved for 30 days. After this time, high titres of virus (6×10^5–1.5×10^7PFU) were recovered from the feet of the VV-HA-TK inoculated mice but not from mice given VV-HA-IL2. This suggested that the IL2 produced by the recombinant virus enabled immunodeficient mice to control the virus infection. The kinetics of viral clearance from the feet of CBA/H mice were not significantly different for VV-HA-TK and VV-HA-IL2 (FIG. *7a*). However, in nude mice, although titres of both VV-HA-TK and VV-HA-IL2 were high at day 3, indicating comparable rates of replication, VV-HA-IL2 was cleared by day 15 when no virus was detected in the feet. Titres of VV-HA-TK still remained high at day 15 (FIG. *7b*). Furthermore, when nude mice were injected intravenously (i.v.) with 10^6 PFU of VV-HA-IL2 or VV-HA-TK, mice given VV-HA-IL2 appeared unaffected by the virus whereas all mice given VV-HA-TK were moribund by day 15. Consistent with this result infectious virus was recovered from spleens and lungs of VV-HA-TK- but not VV-HA-IL2-infected mice (Table 1).

TABLE 1

Vaccinia Virus recovered from Lungs and Spleen			
Mouse Strain	Organ	Log.10 VV-HA-TK	Log.10 VV-HA-IL2
CBA/H	Lung	<2.0	<2.0
	Spleen	<2.0	<2.0
Outbred	Lung	4.91 ± 0.27	<2.0
Nude	Spleen	3.64 ± 0.11	<2.0

Lungs and spleens were collected from 3–4 mice/group 11 days post i.v. inoculation with 10^6 PFU of the indicated virus.

Methods

(a) Murine IL-2 cDNA was subcloned from pcD-IL-2, (14) generously provided by Dr K. I. Arai, DNAX Research Institute, Palo Alto, Calif. VV-HA-IL2 and VV-HA-TK were constructed by insertion of the HSV TK gene plus a chimeric promoter-IL2 fragment or alone, into the HindIII F region of the recombinant VV-HA (previously described as VV-PR8-HA6) (26) using plasmids as described in Example 1 and appropriate selection protocols (27, 28).

(b) 143B cells 2×10^6 were infected with VV-HA-IL2 at 5 PFU/cell. The supernatants (1.5 ml), harvested at the time points indicated, were assayed for IL2 activity using CTLL-2 cells (29) and the calorimetric method for cell growth of Mosmann (30). The results are presented as the log dilution of supernate producing 50% of maximum proliferation in the cell cultures.

EXAMPLE 3

A vaccinia virus expressing IL-3 was constructed as shown in FIG. 2, using the methods described in Example 1.

Irradiated mice (650 Rads) injected with vaccinia virus (10^6PFU intravenously) expressing IL-3 (VV-IL-3) show a reconstituted haematopoietic system within seven days. Spleen cell counts are given below:

	Spleen Cell Number	
	VV-IL-3	NIL
4 days	3×10^7	5×10^6
7 days	2×10^8	2×10^6
10 days	1×10^8	3×10^6

In addition, the injection of vaccinia virus expressing IL-3 (VV-IL-3) protects mice against the lethal effects of irradiation, as follows:

	Deaths	
Irradiation Dose	VV-IL-3	NIL
950 Rads	0/6	6/6

EXAMPLE 4

This example describes the construction of a recombinant adenovirus expressing interleukin genes.

The starting virus for the adenovirus construct is adenovirus type 5 deletion mutant d1 327 that lacks the Xba fragment from 78.5 map units to 84.7 map units in early region 3 (31). This deletion mutant allows the insertion of DNA without exceeding the amount of DNA that can be included in the virus particle. The removal of the E3 region also prevents production of a virus protein that complexes with the major histocompatibility heavy chain protein and reduces the cell-mediated immune response to the virus. The Bam fragment from 60 map units to the right hand end of the viral DNA is cloned in plasmid. The plasmid DNA is cut downstream of the E3 promoter with a suitable restriction enzyme and the interleukin gene inserted in place of the original E3 gene, under the control of the natural E3 promoter. The resulting plasmid containing the interleukin gene in the 60 to 100 map unit fragment of d1 327 is cut with the appropriate restriction enzyme to separate viral and plasmid DNA and transfected into cells together with the overlapping EcoR1 A fragment (0 to 76 map units) of wild type virus. Recombination between the two overlapping DNA fragments will reconstitute viable adenovirus in which the E3 gene is replaced by the interleukin gene.

EXAMPLE 5

FIG. 8 outlines the construction of human immunodeficiency virus IL-2 recombinant vaccinia virus in accordance with the present invention. pFB-X/IL-2 is constructed as shown using the methods described in Example 1. The construction of pTG1125 is as previously described (33). As shown in FIG. 8, plasmid pTG 1125 is transfected into the vaccinia TK gene to give recombinant vaccinia virus VV-HIV, and the plasmid pFB-X/IL-2 is then transfected into VV-HIV to give the desired recombinant vaccinia virus in accordance with this invention.

EXAMPLE 6

(a) This example demonstrates protection from influenza virus A/PR/8/34 in nude mice recovered from infection with VV-HA-IL2.

Groups of 10 Swiss outbred nude mice were inoculated intravenously with VV-HA-IL2 (see Example 2) at various doses and a control group was not given any virus. After 30 days, all mice were challenged with $10LD_{50}$ of A/PR/8/34 intranasally and mortality recorded. The results are shown in FIG. 9, and show that nude mice that are immunised with VV-HA-IL2 survive the vaccination and develop an immune response that confers some protection against a challenge with influenza virus.

(b) The following Table sets out results demonstrating that VV-HA-IL2 also offers better survival from immunisation with a recombinant vaccinia virus, as compared to VV-HA-TK which does not express IL2, in another model of immunodeficiency, sublethally irradiated (700R) mice:

Mortality and mean time to death in sublethally irradiated mice after intranasal inoculation.

	% Survival[a]		MTD[b]	
Experiment	VV-HA-TK	VV-HA-IL2	VV-HA-TK	VV-HA-IL2
1	0	88	11.0	13.0
2	0	100	11.4	—
3[c]	0	43	10.0	21.5
4	0	57	11.2	14.0
5	0	70	10.6	15.3

[a)]Mice were inoculated in. with 10^7 pfu virus within 1 hour of irradiation and were monitored for 21 days or until all animals were dead or had recovered.
[b)]Mean time to death.
[c)]BALB/c mice; CBA/H mice were used in the other experiments.

(c) The following Table sets out results showing that in sublethally irradiated CBA/H mice, immunisation with VV-HA-IL2 confers some protection against a subsequent challenge with A/PR/8/34 influenza virus (see Experiment 1). When the dose of vaccinia virus used for immunisation was lowered to allow mice to survive immunisation with viruses that did not express IL2, it can be seen that VV-HA-IL2 does not confer better protection against influenza virus than VV-HA-TK (see Experiment 2) indicating that IL2 is not increasing the protective immunity, but is allowing the immunodeficient mice to survive a full dose of the vaccine and the surviving mice do have protective immunity. Experiment 3, again with a lower dose of virus, demonstrates that the recombinant viruses do provide protective immunity against subsequent challenge with vaccinia virus.

Protective immunity in VV-HA-IL2-recovered, sublethally irradiated mice.

Immunizing Virus[a]	Challenge Virus[b]	% Survival	MTD[c]	Morbidity
Experiment 1				
10^7 pfu VV-HA-IL2	100 LD_{50} A/PR/8/34	71	11.5	+
NIL	100 LD_{50} A/PR/8/34	0	6.6	++
10^7 pfu VV-HA-IL2	10 LD_{50} A/PR/8/34	86	9.0	+
NIL	10 LD_{50} A/PR/8/34	0	8.7	++
Experiment 2				
10^5 pfu VV-HA-IL2	10 LD_{50} A/PR/8/34	80	9.0	+
10^5 pfu VV-HA-TK	10 LD_{50} A/PR/8/34	80	9.5	+
10^5 pfu VV-KD-B2M	10 LD_{50} A/PR/8/34	22	9.0	++

-continued

Protective immunity in VV-HA-IL2-recovered, sublethally irradiated mice.

Immunizing Virus[a]	Challenge Virus[b]	% Survival	MTD[c]	Morbidity
Experiment 3				
10^5 pfu VV-HA-IL2	10^8 pfu VV-WR	100	—	+/−
10^5 pfu VV-HA-TK	10^8 pfu VV-WR	100	—	+/−
NIL	10^8 pfu VV-WR	10	8.7	++

[a)]Groups of 7–10 sublethally irradiated mice were immunized in. with the indicated virus.
[b)]Mice were challinged with A/PR/8/34 (in.) or VV-WR (iv.) 3 weeks after immunization.
[c)]Mean time to death.

EXAMPLE 7

This example demonstrates that IL2 expressed from a separate virus that infects the same site, but not necessarily the same cell, does not clear virus as efficiently as when all virus expresses IL2. That is, it is important that the IL2 is co-expressed by the virus so that it can be most efficiently delivered.

CBA/H mice were inoculated into a hind footpad with 10^7 pfu VV-HA-IL2 or 10^6 pfu VV-HA-TK or a mixture of the two viruses. Feet were removed 15 days later and assayed for virus. The results are shown in the following Table:

VIRUS	CLEARED TOTAL/(%)
VV-HA-IL2 + VV-HA-TK	2/13 (15.4)
VV-HA-IL2	10/18 (55.6)
VV-HA-TK	1/14 (7.1)

EXAMPLE 8

This example demonstrates the augmentation of vaccinia virus-specific IgA by IL5, and shows that IL5 increases the secondary IgA levels of antibody that are specific for vaccinia virus. The increase in total antibody probably reflects the increase in IgA and other data indicate that other classes of antibody are not increased by IL5.

Antibody was assayed by ELISA, using whole vaccinia virus as the antigen. A serum containing vaccinia-specific antibody was used to construct a standard curve with the vaccinia-specific antibody titre expressed as arbitrary units.

CBA/H mice were inoculated intravenously with 10^7 pfu vaccinia virus and bled 14 days later for primary antibody. On day 28 after primary inoculation, the mice were re-inoculated with the same dose of virus and were bled 7 days later for assay of secondary antibody. In the following table, mean titres of groups of 5 mice are shown with the range of values in brackets.

VIRUS	IgA	TOTAL ANTIBODY
	Primary	
VV-IL5	444	1255
	(<125–1447)	(733–1846)
VV-HA	309	522
	(<125–1200)	(469–856)

-continued

VIRUS	IgA	TOTAL ANTIBODY
	Secondary	
VV-IL5	31,550 (6,500–100,000)	56,436 (18,350–95,238)
VV-HA	5,155 (800–11,400)	40,254 (10,825–98,039)

VV-HA has been described previously (26). VV-IL5 was constructed from plasmid pEDFM-5 (obtained from Dr. I. Young, John Curtin School of Medical Research, Australian National University) containing the murine IL-5 sequence (45). The plasmid was cut with HinPI at sites 24 and 650 of the gene and cloned into the Acc I site of pBCB07 (25,38). The EcoRI fragment with the p7.5 promoter was then cloned into the EcoRI site of pFBX. pFBX-IL-5 was then marker rescued into VV-PR8-HA6 to make VV-IL5.

EXAMPLE 9

This example demonstrates that by inclusion of the gene for murine IFN-γ in a recombinant vaccinia virus (VW), marked attenuation and reduction in pathogenicity can be achieved. VV infection is usually lethal in immunodeficient animals, however it has been found that both athymic nude mice and sublethally irradiated euthymic mice could resolve an infection with VV expressing IFN-γ.

FIG. **10** shows the survival of athymic Swiss outbred nude mice infected with W recombinants (7 mice per group).

FIG. **11** shows the survival of sublethally irradiated CBA/H euthymic mice infected with W recombinants (VV-FB-TK, VV-muIFN-γ-TK: 7 mice per group; VV-huIFN-γTK: 6 mice per group; uninfected: 5 mice per group).

MATERIALS AND METHODS

a. Construction of VV-μIFN-γ-TK and VV-μIL2-IFN-γ.

The murine IFN-γ (pIFN-γ) cDNA clone (36), in the pcD expression vector, was kindly provided by DNAX Research Institute, Palo Alto, Calif., USA. The whole coding sequence between nucleotide positions 1 and 870 was cut out using Sau 3A and Rsa I and ligated into the Bam HI and Hinc II sites of the pBCB07 vaccinia expression vector (25,38). This plasmid was further digested with Eco RI and the fragment containing the P7.5 vaccinia promoter and the μIFN-γ cDNA coding sequence between positions I and 706 was then ligated into the Eco RI site of pFB-TK (27). The recombinant plasmid could then be used in a marker rescue with a VV-WR-TK$^-$ mutant (originated from Dr. B. Moss, NIH, Bethesda, Md., USA) and with VV-IL2, a thymidine kinase (TK) negative recombinant (39), which contains the IL2-expressing gene in the J region of the vaccinia strain VV-WR, interrupting the VV-TK gene. Subsequent plaque purification under methotrexate selection (27) produced two recombinant viruses, VV-μIFN-γ-TK and VV-μIL2-IFN-γ, both of which contained the μIFN-γ gene and the herpes simplex virus (TK) gene in the F region of vaccinia. In subsequent immunological assays the recombinant virus VV-FB-TK (27), was used as a control together with VV-HA-IL2 (see Example 2). In some assays another control virus, VV-huIFN-γ-TK, containing the human IFN-γ gene, was included. The cloning of this CDNA into the pBCB07 vector was carried out as previously described (40) and the virus was constructed through pFB-TK cloning in the same way as VV-μIFN-γ-TK.

b. Bioassay for murine IFN-γand flow cytometry.

Tissue culture supernatants (2.5 ml) of VV-infected human 143B cells (2x10^6 were treated as previously

described (40). Induction of MHC antigens was tested on BALB/c mouse embryo fibroblasts (MEF) (41) and a murine myeloid tumour cell line WEHI-3B (42) using flow cytometry (FACS IV; Becton Dickinson). For MEF, 1 ml of each supernatant was added into 1x10^6 cells/3 ml medium, and for WEHI-3B, 2 ml of supernatant was added into 0.5x10^6 cells/2 ml medium (both subconfluent on 5 cm petridishes). Recombinant murine IFN-γ (rIFN-γ), provided by Boehringer Ingelheim (Vienna, Austria), was used as a positive control. The cells were labelled as previously described (41,40). Monoclonal antibodies against K^d/D^d (HB-79) and Ia^d (HB-3) were obtained from American Type Culture Collection.

c. Mice.

CBA/H (H-2^k), BALB/c (H-2^d) and athymic Swiss outbred nude mice were bred at the John Curtin School of Medical Research, Australian National University, under specific pathogen-free conditions.

RESULTS

a. Bioassay of VV-expressed murine IFN.

Tissue culture supernatants, harvested 12–14 h after infection of human 143B cells with VV (5 pfu/cell), were tested for their ability to induce MHC class I and class II antigens on MEF and WEHI-3B cells, respectively. After 48 h of incubation, VV-μIFN-γ-TK supernatant caused a clear increase in MHC expression on MEF, whereas VV-FB-TK and VV-huIFN-γ-TK supernatant treated cells expressed the same levels of MHC as those without any treatment. For WEHI-3B, untreated control cells showed two peaks, one of which disappeared with rIFN-γ, W-μIFN-γ-TK and VV-μIL2-IFN-γ supernatant treatments, indicating induction of MHC class II antigens. Again, WV-FB-TK and VV-huIFN-γ-TK supernatant treated cells were identical with the control treated cell population. Thus, the IFN-γ secreted by WV-μIFN-γ-TK and μIL2-IFN-γ infected cells was biologically active on murine but not on human 143B cells, where class I expression could be induced with human rIFN-γ or VV-huIFN-γ-TK supernatant (data not shown).

b. Infection of immunodeficient athymic nude mice and sublethally irradiated euthymic mice with recombinant vaccinia viruses.

9-week old athymic nude mice were injected i.v. with 5x10^6 pfu of VV recombinants and their survival monitored daily (FIG. **10**). The mean survival of mice injected with control virus (VV-FB-TK) was 8.4 days, whereas all VV-μIFN-γ-TK, VV-μIL2-IFN-γ and VV-HA-IL2 infected mice survived infection and lived for several months. For VV-huIFN-γ-TK, one mouse recovered from infection and the mean survival for the rest was 21.7 days.

9-week old CBA/H euthymic mice were given a sublethal dose (650R) of gamma irradiation and then injected i.v. with each of the recombinant VV (107 pfu). Again, all VV-FB-TK and all except one VV-huIFN-γTK infected mice died early after infection (mean survival 7.7 days and 11.2 days respectively). Only one VV-μIFN-γ-TK infected and one uninfected control mouse died, but the rest survived for the several weeks of the experiment (FIG. **11**).

c. Recombinant virus growth in mouse organs.

7-week old female CBA/H mice were injected i.v. with 10^7 pfu of VV recombinants. Groups of four mice were killed daily and their ovaries collected for virus titration. Ovaries were chosen for analysis because it has previously been shown that they are a very sensitive indication of attenuated virus growth. VV-FB-TK grew to very high titres in the ovaries reaching titres of $10^{8.2}$ by day 3; however, by day 10 almost all the virus was cleared. VV-huIFN-γ also grew to high titres but was cleared slightly faster than

VV-FB-TK. In contrast, the peak virus titre reached with VV-μIFN-γ-TK and VV-μIL2-IFN-γ was $10^{4.8}$ and $10^{2.8}$, respectively, on day 2, and by day 4 the mice had completely resolved the infection.

EXAMPLE 10

This example demonstrates the construction of a recombinant vaccinia virus which encodes the gene for murine tumour necrosis factor (TNF)-α, and shows that the localised production of TNF-α during a viral infection leads to the rapid and efficient clearance of the recombinant virus in normal mice and attenuates the otherwise lethal pathogenicity of the virus in immunodeficient animals.

FIG. 12 shows:

(a) Genomic configuration of VV recombinants. A HindIII map of VV strain WR (VV-WR) is shown with insertion points at the BamHI (B) and EcoRI (E) sites in the F and J regions respectively. Arrows indicate orientations of VV thymidine kinase gene (vvTK), VV promoters (P7.5 and PF) and inserted genes. VV-PR8-HA6 (described previously as VV-HA), a L929 cell line adapted strain of VV-WR containing the haemagglutinin gene of influenza virus, A/PR/8/34 in the J region, was used to construct the recombinant viruses used in this study. The recombinant virus VV-HA-TNF contains within the F region the whole CDNA coding sequence including signal peptide for murine TNF-α (CDNA supplied by Prof. W. Fiers, State University of Ghent, Belgium) under the control of the VV 7.5 kDa promoter, p7.5 (provided by Dr. B.Moss, NIH), along with the thymidine kinase gene of herpes simplex (HSV-TK) which was used as a selectable marker. The control virus, VV-HA-TK (described previously), similarly contains the HSV-TK gene but not TNF-α cDNA in the F region.

(b) Assay for expression of biologically active TNF by VV-HA-TNF-infected cells. Confluent monolayers of the human osteosarcoma cell line, 143B were infected with the VV recombinants at 5 pfu/cell in 6 well multidishes. At the indicated time points, the supernatants from duplicate wells were harvested, filtered through 0.2 μm filters twice and frozen. Samples were assayed using the TNF-sensitive fibrosarcoma cell line WEHI164 treated with 2 μg/ml of Actinomycin D, and a colorimetric method to quantify cell death using the tetrazolium salt, MTT. TNF units were calculated from a standard curve generated with serially diluted recombinant human TNF-α (Chiron, USA, specific activity= 5×10^7 Units/mg).

FIG. 13 shows the attenuation of the VV-HA-TNF in vivo:

(a) Survival study of immunodeficient mice inoculated with VV-recombinants. Groups of 9 week old Swiss outbred nude and 8 week old sublethally (S/L) irradiated mice were inoculated intravenously (i.v.) with the recombinant viruses at a dose of 5×10^6 pfu and 1×10^7 pfu respectively. Mortality of VV-HA-TK infected nude (—●—) and S/L irradiated (- - - ● - - -) mice and the survival of VV-HA-TNF infected nude, VV-HA-TNF infected S/L irradiated and uninfected control mice (—O—) are shown.

(b), Growth kinetics of the VV recombinants in normal mice. Groups of 9-week old female CBA/H mice were injected i.v. with a non-lethal dose, 10^7 pfu, of either VV-HA-TNF or VV-HA-TK. On the indicated days, selected organs were collected for titration of virus on 143B cell monolayers. Error bars indicate standard errors of the mean titre for groups of 4 mice.

The recombinant viruses (VV-HA-TNF and VV-HA-TK) used in this study were constructed using VV vectors and homologous recombination and selection methods as described previously (43) (FIG. 12*a*). High levels of TNF were detected in vitro following infection of 143B cell monolayexs with the VV-HA-TNF virus (FIG. 12*b*), indicating effective vector-directed expression and secretion of biologically active TNF as measured by a cytotoxicity assay (44).

In order to compare the in vitro replicative efficacy of VV-HA-TNF to that of the control virus, VV-HA-TK, a single-step growth experiment (multiplicity of infection (MOI)=5 pfu/cell) was performed. Under these conditions, growth profiles for both recombinant viruses were similar in either CV-1 (simian) or L929 (murine) cell lines (data not shown). This indicates that the ability of VV-HA-TNF to replicate in vitro at high MOI, is not altered by insertion of the TNF gene or by the expression of TNF. To test the sensitivity of VV to the antiviral effects of TNF, the cell lines, L929, 143B, 293, HeLa and primary rat embryo fibroblasts were pretreated with 0.1–400 ng/ml recombinant murine TNF-α (Genentech, specific activity=1.2×10^7 Units/mg, supplied by Boehringer Ingelheim) or human TNF-α (Asahi, specific activity=2.2×10^6 Units/mg) for 24 h and then infected with VV-WR (wild type). When the virus yield was measured 24 h later, only L929 cells showed reduced virus growth (up to 1.5 log) in the presence of TNF. Some toxicity towards the L929 cells was noted, however, at the concentration of TNF required to inhibit virus replication.

In contrast to these in vitro results, which suggest that W is not highly susceptible to the antiviral effects of TNF, expression of TNF markedly attenuated the growth of W-HA-TNF in vivo (FIG. 13). Two models of immunodeficiency were used: athymic Swiss outbred nude mice and euthymic CBA/H mice rendered immunodeficient by a sublethal dose of γ-irradiation (650R) administered 24 h prior to infection. Both the nude and irradiated mice infected with the control virus, VV-HA-TK, died from a disseminated vaccinial disease, with a mean survival time of approximately 10 days (FIG. 13*a*). In contrast, when infected with W-HA-TNF, both groups of mice survived and remained as healthy as the uninfected controls, indicating that TNF expression had reduced the pathogenicity of VV in these mice (FIG. 13*a*).

The attenuating effect of TNF expression on virus growth was also seen in normal mice (FIG. 13*b*). Neither W-HA-TNF, nor its control, VV-HA-TK, produced morbidity or mortality at the doses used. However, VV-HA-TNF was recovered from various organs at significantly reduced titres and the virus cleared more rapidly as compared to VV-HA-TK (FIG. 13*b*). The difference in growth between the two viruses was evident by 24 h post-infection (p.i.) (FIG. 13*b*) and even earlier in some cases (data not shown). Growth in the ovaries provided one of the most prominent indicators of virus attenuation. W-HA-TK grew to very high titres, reaching $10^{8.1}$ pfu per pair of ovaries by day 3, and was cleared only by day 10, whereas VV-HA-TNF reached a mean peak titre of $10^{3.5}$ pfu per pair of ovaries and was cleared 3–5 days p.i. Histological examination of the ovaries from mice infected with W-HA-TK revealed extensive damage to the stromal tissue and follicles, whereas those from VV-HA-TNF infected mice appeared normal (data not shown).

EXAMPLE 11

This example compares the effect of administration of exogenous recombinant IL-2 or recombinant IFN-γ on the growth of vaccinia virus with the effects of co-expressed IL-2 (VV-HA-IL2).

Groups of 3 to 5 outbred nude mice infected i.v. with 10^7 pfu VV-HA-TK were given 600U of rIL-2 or rIFN-γ i.p. every 8 h for a period of 5 days. Morbidity and mortality in these groups and others given virus alone or the recombinant cytokines alone was assessed. No morbidity or mortality was recorded in groups of mice given only rIL-2 or rIFN-γ. All mice given VV-HA-IL2 alone survived with no overt disease. Nude mice infected with VV-HA-TK alone showed signs of disease by 6 days p.i. and all mice died with a MTD of 12.2 days. Treatment with exogenous rIL-2 or rIFN-γ delayed the onset of disease signs which appeared between 11–16 days p.i., and significantly ($p < 0.001$) prolonged survival of nude mice. Nevertheless, all mice that had been infected with VV-HA-TK and treated with either rIL-2 or rIFN-γ succumbed to disseminated disease and died with MTD of 23.4 and 25.8 days, respectively.

The survival rates are summarised in the following Table:

TREATMENT	VV-HA-IL2	VV-HA-TK (Control)
nil	5/5 survivors	0/5 survivors
rIL-2 600 U every 8 hrs for 5 days	ND	0/5 survivors
rIFN-γ 600 U every 8 hrs for 5 days	ND	0/5 survivors

EXAMPLE 12

In this example, a recombinant vaccinia virus (VV-HA-IL5) expressing IL-5 in combination with the haemagglutinin of influenza virus PR8 (HA) is constructed using the methods described in Example 1. Thus, the HA gene was inserted in the J region of vaccinia virus strain WR (see FIG. 2), and the genes for murine IL-5 (45) and thymidine kinase of HSV in the F region.

The leukaemic B cell line BCL_1, which responds to IL-5 by proliferating, was used to test for expression of IL-5 by the construct. FIG. 14 shows that 12 hour supernatants from VV-HA-IL5-infected 143B cells stimulated BCL_1 to proliferate in a dose-dependent manner, unlike those from uninfected 143B cells or from cells infected with the control virus, VV-HA-TK, which encodes HA but not IL-5.

In time-course experiments to assess virus growth in vivo, it was found that both VV-HA-IL5 and VV-HA-TK exhibit similar growth kinetics in murine lung when administered intranasally (FIG. 15).

IL-5 activity in vivo was assessed by examining the influence of VV-encoded IL-5 on the immune response to co-expressed HA in vivo, in terms of numbers of HA-specific antibody-secreting cells (ASC). This parameter was chosen as most likely to reflect IL-5 activity, given existing evidence from in vitro studies that this factor appears to act in promoting terminal differentiation of B cells. The ELISPOT assay was used to enumerate HA-specific antibody-secreting cells. This assay detects specific antibody secreted by each cell as an insoluble spot on nitrocellulose membrane.

No elevation in numbers of anti-HA ASC of IgM, G or A isotypes were detected in either lungs or spleen of mice given VV-HA-IL5 intravenously compared with those given control virus (data not shown). Similarly, IgM or IgG ASC levels in the lung were unaffected following intranasal inoculation. However, following intranasal inoculation, numbers of anti-HA IgA ASC in lungs were up to 4-fold higher in mice given VV-HA-IL5 (FIG. 16—mice given intranasally 10^7 plaque-forming units of virus at day 0). These differences became apparent by day 16 after infection and persisted for over 3 weeks.

In vitro evidence supporting the concept that IL-5 is acting to increase ASC numbers by increasing differentiation of committed membrane IgA-positive cells to plasma cells is shown in the following table. Mice were primed with influenza virus PR8 and lung cells were isolated 14 days later, T-depleted, fractionated by panning and cultured for 4 days. Both unfractionated B cells and $mIgA^+$ cells cultured with recombinant IL-5 displayed increased ASC numbers, unlike the $mIgA^-$ fraction.

TABLE

Effect of rIL5 on IgA ASC numbers in immunised lung.

inoc. in vivo	restim in vitro	Total IgA ASC/10^6 cells cultured: B cells	$mIgA^+$	$mIgA^-$
PR8	none	200 ± 60	1220 ± 220	20 ± 10
PR8	rIL-5 (5 u/ml)	520 ± 125	3750 ± 435	30 ± 12
none	rIL-5	220 ± 80	1590 ± 450	35 ± 20

The above data provides evidence for the ability of IL-5 co-expressed with HA in recombinant VV to augment IgA responses to HA In vivo. As IgA functions in virus neutralisation at the most common point of infection, i.e. the mucosae, such responses may afford increased protection.

REFERENCES

1. Howard, M. et al. J. Exp. Med. 157, 1529–1534 (1983).
2. Baracos, V. et al. New Eng. J. Med. 308, 553–558 (1983).
3. Schmidt, J. A. et al. J. Immun. 128, 2177–2182 (1982).
4. Mizel, S. B. in Biological Response in Cancer (ed. Mihich E.) 89–119 (Plenum, New York 1982).
5. Lomedico, P. T. et al. Nature 312, 458–461 (1984).
6. Auron, P. E. et al. Proc. Natl. Acad. Sci. US 81, 7907–7911 (1984).
7. Smith, K. A. Ann. Rev. Immunol. 2, 319–333 (1984).
8. Minato, N. et al. J. Exp. Med. 154, 750 (1983).
9. Tsudo, M. et al. J. Exp. Med. 160, 612–616 (1984).
10. Merluzzi, V. J. et al. Cancer Res. 41, 850–853 (1981).
11. Wagner, H. et al. Nature 284, 278–80 (1982).
12. Vose, B. M. et al. Cancer Immuno. 13, 105–111 (1984).
13. Rosenberg, S. A. et al. Science 233, 1318–1321 (1986).
14. Yokota, T. et al. Proc. Natl. Acad. Sci. USA 82, 68–72 (1985).
15. Taniguchi, T. et al. Nature, 302, 305–307 (1983).
16. Schroder, T. W. et al. in Contemporary Topics in Molecular Immunology: The interleukins, eds Gillis, S. and Inman, F. P. (Plenum, N.Y.) Vol. 10, pp. 121–146 (1985).
17. Yokota, T. et al. Proc. Natl. Acad. Sci. USA 81, 1070–1074 (1984).

18. Sevenusar, E. Eur. J. Immunol. 17, 67–72 (1987).
19. Lee, F. et al. Proc. Natl. Acad. Sci. USA 83, 2061–2065 (1986).
20. Yokota, T. et al. Proc. Natl. Acad. Sci. USA 83, 5894–5898 (1986).
21. Gray, P. W. et al. Nature 295, 503–508 (1982).
22. Smith, G. L. et al. Nature 302, 490–495 (1983).
23. Hu, S. L. et al. Nature 320, 537–540 (1986).
24. Boyle, D. B. and Coupar, B. E. H. J. Gen. Virol. 67. 1591–1600 (1986).
25. Boyle, D. B. et al. Gene 35, 169–177 (1985).
26. Andrew, M. E. et al. Microbial Pathogenesis 1, 443–452 (1986).
27. Coupar, B. E. H. et al. J. Gen. Virol. 68, 2299 (1987).
28. Coupar, B. E. H. et al. Gene 68, 1–10 (1988).
29. Gillis S. et al. J. Immunol 120, 2027–2032 (1978).
30. Mosmann, J., J. Immunol. Meth. 65, 55–63 (1983).
31. Cutt, J. R. et al. J. Virol. 61, 543 (1987).
32. Wright, D. C. et al, New Eng. J. Med. 316, 673–675 (1987).
33. Kierny, M. P. et al. Biotechnology 4, 790–795 (1986).
34. Andrew, M. E. et al. Scand. J. Immunol. 25, 21–28.
35. Smith, G. L. et al. Proc.Natl.Acad.Sci. 80, 7155–7159.
36. Gray, P. W. et al. Proc.Natl.Acad.Sci. USA, 80, 5852 (1983).
37.
38. Andrew, M. E. et al. J.Virol. 61, 1054, (1987).
39. Mullbacher, A. et al. Scand. J. Immunol., 29, 1, (1989).
40. Kohonen-Corish, et al. J.Immunol., 143, 623, (1989).
41. King, N. J. C. et al. Expl.Clin.Immunogenet., 2, 206, (1985).
42. Wong, G. H. W. et al. J.Immunol., 131, 788, (1983).
43. Ramshaw, I. A. et al. Nature, 329, 545–546 (1987).
44. Espevik, T. et al. J.Immun. Methods, 95, 99–105, (1986).
45. Kinashi, T. et.al.. Nature 324, 70–73 (1986).

We claim:

1. A preparation for stimulating an immune response in a human or animal host comprising a vaccinia virus vector incorporating a first nucleotide sequence capable of being expressed as an antigenic polypeptide which is foreign to the host vector, together with a second nucleotide sequence capable of being expressed as a polypeptide having lymphokine activity selected from the group consisting of interleukin-2, interleukin-3, interleukin-4, interleukin-5, γ-interferon and tumour necrosis factor, and which is effective in enhancing the immune response in the human or animal host to the antigenic polypeptide when compared to the immune response in the human or animal host administered a vaccinia virus vector incorporating only the first nucleotide sequence.

2. A method for the production of the preparation according to claim 1, which comprises the step of inserting into a vaccinia virus vector a nucleotide sequence capable of being expressed as a polypeptide having lymphokine activity selected from the group consisting of interleukin-2, interleukin-3, interleukin-4, interleukin-5, γ-interferon and tumour necrosis factor, said method further comprising inserting into the vector a nucleotide sequence capable of being expressed as an antigenic polypeptide which is foreign to the host.

3. A method for producing an immune response in a human or animal which comprises the step of administering to the human or animal a preparation according to claim 1.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : **5,866,136**
DATED : **Feb. 2, 1999**
INVENTOR(S) : **Ian A. Ramshaw et al.**

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, Item [73],

The Assignees should read : --Commonwealth Scientific and Industrial Research Organisation and The Australian National University--

Signed and Sealed this

Sixth Day of July, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks